Effect of Lees Manipulation on the Chemical and Sensorial Characteristics of New Zealand Sauvignon blanc Wine

---

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy

at

Lincoln University

by

Zhijing (Victor) Ye

Lincoln University

2020

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

Effect of Lees Manipulation on the Chemical and Sensorial Characteristics of New Zealand Sauvignon blanc Wine

by

Zhijing (Victor) Ye

Sauvignon blanc wine currently comprises about 86% of total NZ wine exports. Its success has been ascribed to its distinctive flavour and aroma, which has been the subject of much research. However, less attention has been paid to the role of palate structure (e.g. mouthfeel) in defining the style, and to the effects of specific winemaking techniques such as lees contact during wine aging.

In this study, the effects of different lees management techniques (lees type, stirring frequency, and addition of β-glucanse) on the release of chemical compounds (i.e. nitrogenous compounds, polysaccharides, mannoprotein, β-glucan and glycerol) in model wine were investigated. Because yeast autolysis is a slow process at low temperatures and at the low pH values existing during winemaking, the study also investigated the release of chemical compounds from pulsed electric field (PEF) induced yeast autolysis using rehydrated active yeast with/without addition of β-glucanse. In addition to limited information on lees management appropriate to New Zealand styles of Sauvignon blanc, the lexicon to describe Sauvignon blanc wine mouthfeel is poorly investigated. In this study, napping® was used in order to try and capture terms for the description of mouthfeel in Sauvignon blanc wine, and to determine how these words relate to the chemical composition of wines.

Time and lees materials had a significant impact on the concentrations of total protein, primary amino acids nitrogen (PAN), neutral and acidic polysaccharides, mannoprotein, β-glucan and glycerol in model wines ($P < 0.001$). In addition, inactivated yeast was a good source of amino acids, especially Pro, Arg, Glu and Gln. Inactivated yeasts was a better source of polysaccharides than rehydrated yeasts

and collected lees. The majority of the polysaccharide released was in the neutral form and reached concentrations ranging from 600 to 800 mg galactose/L on day 160 in model wine. Both addition of β-glucanases and stirring frequency were minor factors that affected the release of chemical compounds from lees material. Stirring frequency had a significant impact on the concentrations of protein, PAN and neutral polysaccharide ($P < 0.05$). In addition, stirring frequency showed a statistical impact on protein, PAN and polysaccharide in model wines, but differences were small compared with the overall changes that were achieved at the end of the aging experiment.

The study also showed it was feasible to accelerate on-lees aging with pulsed electric field. The experiment demonstrated that the application of PEF (5.5 and 10.0 kV/cm field strength) to lees (5% w/v, rehydrated yeasts) provoked a release of intracellular materials, such as nitrogenous compounds, polysaccharides, and mannoprotein. In addition, time and lees materials also had a significant impact on the concentrations of total protein, primary amino acids nitrogen (PAN), neutral and acidic polysaccharides, mannoprotein, β-glucan and glycerol in model wines ($P < 0.001$).

In the current study, perceived sweetness and acidity seemed to be the major criteria for sorting the commercial examples of Sauvignon blanc wines. In total, 253 descriptors were collected but more than half of these descriptors were used no more than twice on different occasions, and a combination of synonyms was not able to reduce this number significantly. Descriptors that had a frequency of more than 20 were too few to create a comprehensive lexicon. There was no precise correlation between chemical composition and collected descriptors (especially for the ones with low frequency). However, the distribution of wines on the consensus map seemed to be associated with the vinification technique, namely, on-lees aging and vessels used during fermentation and post fermentation. Panellists were able to distinguish the standard and on-lees aged Sauvignon blanc wines.

Key words: β-glucanase, lees, mouthfeel, Napping®, pulsed electric field, stirring frequency.

Acknowledgements

Firstly, I would like to thank my supervisor, Dr Roland Harrison for creating this interesting project and giving me the opportunity to work on it. I really appreciate his patience, time, guidance and advice. I would also like to thank my associate supervisors Dr Alaa El-din Bekhit and Dr Sue Mason for their expertise, time and guidance throughout the project. Alaa El-din has been always there on skype or the email, ready to help me whenever I have a problem; while I was working in the lab in Dunedin, he was always there with me to providing help and guidance. Thank you to Sue who put a lot of effort into helping set up and run sensorial session in Blenheim. I also acknowledge the contributions of Jenny Zhao who trained me in the use of HPLC for analysis of free amino acids; and, the contribution of Joy Jiao for helping the development of the method of mannoprotein quantification; and the contribution of Richard Hider for the consultation of the specific chemical analysis.

I also extend my personal thanks to Mr Andy Frost for kindly providing lees samples and information about winemaking background. I would like to thank Dr Damian Martin and Miss Claire Grose for providing the facility, helping set up and organizing panellists - without your help the sensory test would not be able to run smoothly and successfully.

My greatest gratitude goes to my parents, Jinyou Wang and Lun Ye; I would not have been able to complete my PhD successfully without their help, spiritually and financially. Thanks to their encouragement during my hardest period of study, as well as look after my daughter April while I was working at the University at night. I would like to express my appreciation to my wife Jinlin Shi for her efforts on looking after April and family, even though we had have been through the hardest time in our life. I would like to thank my cousin, Jiong Wang who opened the door to the data science for me; he not only helped me with MatLab programming but also shown me how to become a fit and healthy scientist.

This research could not have been conducted without the financial support from The New Zealand Institute for Plant and Food Research. This study was funded in part by The New Zealand Institute for Plant and Food Research Through their Strategic Science Investment Fund which was previously the Foundation for Research Science and Technology Programme C11X1005. I acknowledge their partnership in the project and would like to thank them for their contribution.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

%	percent
^{o}C	degree Celsius
<	less than
>	more than
=	equal to

Amino acids

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
Cys	cysteine
Glu	glutamic acid
Gln	glutamine
Gly	glycine
His	histidine
Ile	isoleucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan

Tyr	tyrosine
Val	valine
ANOVA	analysis of variance
APS	acidic polysaccharide content
DM	dry matter
e.g.	*exempli gratia* (for example)
et al.	and others
Eth/w	50% aqueous ethanol
FD	freeze-dry
g	gram
GA	gallic acid
GAE	gallic acid equivalent
HPLC	high performance liquid chromatography
HPLC-ELSD	high performance liquid chromatography-evaporative light scattering detector
Hz	herts
hr	hour
i.e.	*id est* (that is)
IDY	inactivated dry yeasts
kg	kilogram
kV/cm	field strength, kilo volts per centimetre
L	collected lees
M	molar
mg	milligram
min	minute

mL	millilitre
mm	millimetre
NI	non-PEF treated inactivated dry yeasts
NR	non-PEF treated rehydrated yeasts
NPS	neutral polysaccharide content
OD	optical density
OH	hydroxide
ppm	parts per million
PAN	Primary amino acid nitrogen
PCA	principle component analysis
PEF	pulsed electrical field
PH	rehydrated yeasts treated with high PEF intensity
PL	rehydrated yeasts treated with low PEF intensity
RNA	ribonucleic acid
R	rehydrated yeasts
rpm	revolution per minute
TPC	total phenolic content
TPS	Total polysaccharide content
TTC	total tannin content
µg	microgram
µM	micromolar
µL	microlitre
µm	micrometre
UV	ultraviolet
V	Volt

v/v	volume/volume
v/w	volume/weight
var.	variety
w/v	weight/volume
w/w	weight/weight

Chapter 1

Introduction

1.1 New Zealand Sauvignon blanc

New Zealand is considered a 'New World' wine producing country. In 2019, 90% of New Zealand produced wines were exported mainly to US, UK, Australia, and Canada (New Zealand Wine Grower, 2019). Such great success for New Zealand wine sales in international markets is largely based on Sauvignon blanc, which contributes to about 86% of total NZ wine exports (New Zealand Wine Grower, 2014). Sauvignon blanc wine has vegetative aromas including: red capsicum, gooseberry, passion fruit, crisp and fresh cut grass notes, and is appreciated by consumers worldwide. Different portfolios of Sauvignon blanc (from standard, select and premium) are made to meet the demands of consumers using different vinification techniques. Sauvignon blanc has attracted the interest of many New Zealand researchers. Specifically, the distinctive flavour of Sauvignon blanc has been investigated from vineyard to glass with the focus on chemical characterization of compounds contributing to the flavour and identification of their origin and precursors. The vegetative aroma in Sauvignon blanc has been mainly attributed to the high concentration of methoxypyrazine in grapes (Allen et al., 1991) due to the cool climate of New Zealand. Less attention has been paid to the role of palate structure (e.g. mouthfeel) in defining the style and on the effects of specific winemaking techniques such as lees contact during wine aging.

1.2 Wine lees

1.2.1 Definition of lees

Lees is an old English word for the dregs or sediments that forms at the bottom of a fermentation vessel (Robinson, 1994). Dead yeast cells and yeast debris are major components of lees, although wine lees is a complex mixture that may also contain tartaric crystals, plant cell debris, polyphenols adsorbed to proteins, lipids and many other compounds (Charpentier, 2010). Lees can be divided into two groups according to particle size: heavy lees which settle within 24 hours after alcoholic fermentation and have particle sizes ranging from 100 µm to 2 mm; and, light lees which settle after more than 24 hours with a particle size from 1 µm to 50 µm (Charpentier, 2010). Lees material can also be classified into three categories according to nature and integrity of yeast cells, such as yeast lees, yeast products (e.g. inactivated dry yeasts) and rehydrated yeasts. In the wine industry, on-lees aging is performed with yeast lees which is generated during wine fermentation, although

inactivated dry yeasts are sometimes added to wine in order to improve sensorial characteristics, e.g. increase mouthfeel and roundness sensations, decrease astringency and increase the aromatic persistence (Del-Barrio-Galá et al., 2011). Rehydrated yeasts are most often used in laboratory studies of on-lees aging, being added to a model wine system to investigate yeast autolysis and subsequent release of chemical components (Guilloux-Benatier & Chassagne, 2003; Rowe et al., 2010).

1.2.2 Winemaking with on-lees aging

Aging on lees, termed *sur lie* in French, is a traditional winemaking practice originally applied to selected white wines from Burgundy as well as sparkling wine using traditional methods. This technique has increased in popularity for use in wines from other regions and also for red wines (Charpentier, 2010). The EEC regulations (No. 822/87) permit the addition of up to 5% of fresh undiluted lees from a recent fermentation of dry wine.

Pérez-Serradilla and Luque de Castro (2008) and Cardini (2006) reviewed the enological function of lees in wine production which includes: the removal of undesirable compounds (e.g. ochratoxin A), interaction with phenolic compounds, inhibition of tartrate salt crystallization, prevention of haze formation, reinforcement of aromatic components, and modification of polysaccharides.

However, aging wine on lees encourages early development of malolactic fermentation, and also increases the risk of off-odour production, such as the formation of hydrogen sulphide, mercaptans or disulphides (Jackson, 1998; Robinson, 1994). Off-odours are likely to arise in wine left in contact with a high concentration of lees for more than a week. There are three different treatments that can minimize these negative effects. Firstly, large particulate lees (between 100 µm and 2 mm) should be removed by racking new wine before aging (Robinson, 1994). Secondly, lees from fresh wine can be stored in a stirred vat for 1 to 2 months before adding back (Chapentier, 2010). Thirdly, periodic stirring of the lees to resuspend the settled lees (a technique called *bâtonnage* in French can be used) (Jackson, 1998). Lees stirring can be done manually with a clean stainless-steel stirring rod or automatically using an agitator. Stirring is normally applied several times per week. Tanks used in sparkling wine production have built-in agitators and temperature control system, which enable fermentation to occur more rapidly than in-bottle (Buxaderas & López-Tamames, 2012). However, the stirring rates and intensity of stirring have not been reported in the literature.

In general, aging on lees is a vinification technique that can achieve a target wine style by enhancing structure and mouthfeel, giving extra body and increasing the aromatic complexity (Charpentier, 2010). During lees contact, yeast components are released as a consequence of an enzymatic cell

self-destruction called yeast autolysis. Among those components released, mannoproteins, amino acids, protein, and nucleotides are responsible for wine mouthfeel, aroma, sweet and bitter tastes, and flavour, respectively (Alexandre & Guilloux-Benatier, 2006). The release of glucans, lipids, protein, and peptides are also responsible for the foaming quality of Champagne and sparkling wine. On-lees aging is increasingly used in bulk storage to improve the flavour of everyday white wine (Robinson, 1994).

Among the sensorial effects of on-lees aging, great emphasis has been placed on improvements to mouthfeel (Rodríguez-Bencomo et al., 2010). In general, the term "mouthfeel" refers to the tactile perception in the oral cavity which includes astringency, touch, viscosity, burning sensation, temperature, body, prickling and pain (Pickering & Demiglio, 2008; Jackson, 2008). Characterization of sensory attributes related to mouthfeel is quite difficult due to lack of an agreed lexicon and supporting scientific data. There is confusion about words that are used to describe the mouthfeel of wine. For example, bitterness, a taste sensation, is also used to describe the mouthfeel at the end of the perception of astringency. Body and weight are also perceptions of mouthfeel, but are often used as parameters in sensory analysis separate from other mouthfeel parameters such as astringency. In fact, the precise origin of the body remains unclear (Jackson, 2008). Scientific research had been carried out to explore the relationship between wine chemical composition and mouthfeel. However, most of the published data is based on red wine.

Aging wine on lees can be carried out in various types of containers including bottle, tank, vat and oak barrel (Robinson, 1994). Traditionally, sparkling wines are stored on lees in bottles for a second fermentation for a minimum time of 9-12 months. In bulk production, sparkling wine is aged on lees in pressure-resistant tanks for 1 to 3 months (short aging) or up to 6 months (long aging) (Buxaderas & López-Tamames, 2012). Both white and red wine can be left in contact with lees in oak barrels. Margalit (2012) suggested that Chardonnay and Sauvignon blanc should be fermented and aged in new oak barrels for one to two years to give the wine a desirable roasted almond flavour as well as using the yeast lees to protect the wine from becoming over-oaked.

Yeast autolysis and the development of flavour in wine is a slow process. Endogenous enzymes such as pectinases and glucanases (β-1,3-1,6 glucanases) were reported to be able to speed up the yeast autolysis process. Commercial solid or liquid enzymes are normally used, (Canal-Llaubères, 2010). The addition of these enzymes benefits clarification and improves the filterability of the resultant wine (Canal-Llaubères, 2010). Heating can also be used to accelerate yeast autolysis and enrich the wine with glutamic acid, lysine, and arginine. A wide range of temperatures and heating times have been reported in the literature, e.g. 65-70°C for 5 days and 33-45°C for 2 or 3 days (Flanzy et al,

1999). However, inactivation of protease enzymes was observed when the wine was heated at a low temperature (40-45°C) for 2 days (Buxaderas & López-Tamames, 2012).

1.3 Objectives of the current study

Management during on-lees aging is very important for wine quality. Even though lees management techniques are widely used, information of the effects of these techniques and their interaction on the release of chemical compounds is still limited. For example, the application of periodic stirring is substantially based on the experiences of winemakers, and stirring frequencies and intensities remain poorly investigated. In addition, on-lees aging is a slow process because yeast autolysis is highly dependent on the surrounding temperature and pH. Ideal conditions of autolysis has been reported to be pH 5 at 45°C (Charpentier & Feuillat, 1993), but in wine, autolysis conditions are less than ideal (e.g. pH 3 at 13°C). This explains why winemakers have to prolong the wine-lees contact time to months and years. Different methods had been developed to speed up the autolysis process (from 48 to 72 hours) including a rise in temperature, alternate freezing and thawing, pH adjustment, the addition of antibiotics, aeration and starvation. However, these methods can have significant negative impacts on wine quality. Pulsed electric field (PEF) is a novel technology that has been recently applied in winemaking to maximize yield and quality of grape juice at the pressing stage (Praporscic et al., 2007). It is a non-thermal method of food preservation where short pulses of electricity are used to inactivate microorganisms and improve the extraction process. PEF may be a suitable approach to reduce aging time by accelerating the mechanism of breaking yeast cell wall membranes.

The objectives of this study are: 1) to investigate the effects of different lees management techniques on the release of chemical compounds; 2) to determine the feasibility of pulsed electrical field for accelerating on-lees aging process; and, 3) to investigate the lexicon for the description of mouthfeel of Sauvignon blanc wine using napping®, and how these words relate to the chemical composition of wines.

Chapter 2

Literature Review

2.1 Introduction

During yeast autolysis, compounds able to affect organoleptic quality are released into wine (Kocková-Kratochvílová, 1990). These compounds, including mannoproteins, glucans, lipids, proteins, peptides, amino acids and nucleotides, are breakdown products of yeasts, primarily their cell walls. Yeasts are eukaryotic organisms, commonly ellipsoidal or ovoid in shape (Figure 2.1) and vary between 1 and 10 µm in size (Feldmann, 2012). From outside to inside, the yeast cell structure consists of the cell wall, cell membrane, and cytoplasm which contains various intracellular organelles.

The survival of yeasts relies on the presence of intact cell walls; these are rigid structures with an approximate thickness of 100-200 nm. Mannoproteins, β-glucans and chitins are the major components of yeast cell walls (Kollar et al., 1997; Feldmann, 2012). Cell membranes are a second barrier layer and contains the lipid bilayer composed of phospholipids as the major lipid group.

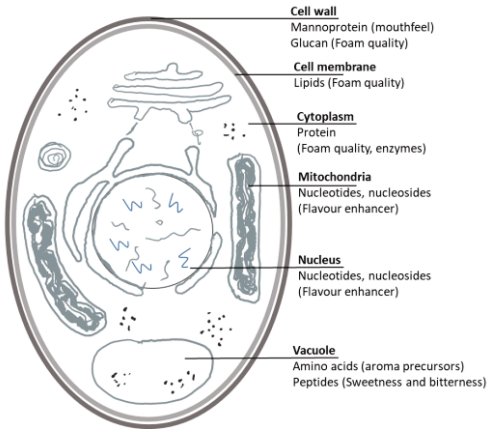


Figure 2.1 Yeast morphology and origin of the different compounds released during autolysis.

Yeast cells contain a number of important structures including vacuole, mitochondria, endoplasmic reticulum (ER), golgi apparatus and nucleolus (Figure 2.1). The vacuole is an important cytoplasmic compartment in the yeast cell containing 80 to 90% of total dissolved amino acids. Mitochondria are the sites of oxidative metabolism which generate ATP in yeast cells. In *Saccharomyces cerevisiae*, the

total mitochondria volume is about 12% of the cell volume. The ER makes up to 10% of the volume of some cells, and is responsible for the synthesis of new proteins and membrane lipids. Golgi apparatus is a stack of flattened membrane sacs which work associated with the ER sorting newly synthesized protein and directing them to their correct cellular destinations (Garrett & Grisham, 2010). In addition, the ER and Golgi apparatus are also the sites at which oligosaccharides are combined with the proteins and which become the essential building blocks of yeast cell walls (Albert et al., 1994).

2.2 Composition of yeast lees

Lees is a complex mixture that may contain water, alcohol, dead yeast materials, plant cell debris, polyphenols adsorbed to proteins, lipids, tartaric acid crystals, and many other compounds (Table 2.1). Rice (1976) reported the moisture content of pressed lees of white and red wine after post alcoholic fermentation to be 52.9% and 54.0%, respectively. These 'wet' lees contained 5.38% (w/w) and 5.41% (w/w) of alcohol. The concentrations and composition of amino acids, nucleotides, β-glucan and mannoproteins in lees are unknown due to limited studies in this area.

Tartaric acid (TA) can be a major component of wine lees. Ye (2014) found TA in lees derived from Riesling and Pinot noir wines ranged from 1.49 to 3.23 and from 1.08 to 5.90 g/kg fresh lees, respectively. This research also suggested that TA content might depend on the vinification technique and grape origin. Most Riesling lees had lower total TA contents than Pinot noir lees, which might be attributed to differences in stabilisation techniques during winemaking. In wine, tartaric acid commonly exists as potassium bitartrate which crystalizes and precipitates during storage at low temperature (Jackson, 1994).

Table 2.1 Summary of elemental composition and main physicochemical characteristics of lees.

Elemental composition	Range	Physicochemical parameter[1]	Range
Ca (g/kg DM)	3.6-15.5	Corg (g/kg DM)	226-376
Cu (mg/kg DM)	13-1187	Cws (g/kg DM)	44.3-168.9
Fe (mg/kg DM)	84-1756	EC(dS/m)	4.0-13.8
K (g/kg DM)	17.6-158.1	OM (g/kg DM)	598-936
Mg (g/kg DM)	0.4-3.7	pH	3.6-7.2
Mn (mg/kg DM)	<0.2-21	Pol (g/kg DM)	1.9-16.3
P (g/kg DM)	1.61-10.3	TN(g/kg DM)	17.2-59.7
Zn (mg/kg DM)	14-84	Proteins (%)	14.5-15. 7
		Lipids (%)	5.0-5.9
		Sugars (%)	3.5-4.8
		Dietary fibre (%)	21.2-21.9
		Tartaric acid (%)	24.5-24.7
		Ash (%)	10.5-10.6

[1]Corg: oxidisable organic carbon; Cws: water-soluble carbon; EC: electric conductivity; OM: organic matter; Pol: water-soluble polyphenols; TN: total nitrogen (Source: Bustanante et al., 2008; Gómez et al., 2004)

Table 2.2 Fatty acids composition of Sherry wine lees.

Fatty acid		Relative amount (%)
Capric acid	C10:0	2.32
Lauric acid	C12:0	4.42
Miristic acid	C13:0	1.98
Palmitic acid	C16:0	33.29
Palmitoleic acid	C16:1	1.80
Margaric acid	C17:0	0.30
Stearic acid	C18:0	10.40
Oleic acid	C18:1	7.82
Linoleic acid	C18:2	21.26
Linolenic acid	C18:3	5.88
Araquidonic acid	C20:0	2.10
Erucic acid	C22:0	6.10
Lignoceric acid	C24:0	2.32

(Source:Gómez et al., 2014)

2.3 Soluble yeast-derived components of importance to palate attributes

2.3.1 Amino acids and peptides

The primary storage site for amino acids in yeast cells is the vacuole (Kitamoto et al., 1988). Wiemken and Dürr (1974) demonstrated that about 60% of the total amino acid pool of *S. cerevisiae* spheroplasts (cells without a cell wall) is contained in the vacuoles. Secondary storage of amino acids is located in the cytoplasm comprising about 12% of the total amino acid pool. The major amino acids in vacuoles are glutamine (Gln), citrulline (Cit), arginine (Arg), lysine (Lys) and ornithine (Orn). In the cytoplasm, aspartic acid (Asp), glutamic acid (Glu) and alanine (Ala) are the major amino acids (Wiemken & Dürr, 1974). Not all soluble amino acids are in free form as some may be partially bound to macromolecules such as polyphosphate. Furthermore, the amino acid concentration in yeast cells is correlated to the nutrients available in their growth medium. Kitamoto et al. (1988) studied the vacuolar and cytosolic amino acid pools extracted from *S. cerevisiae* cells grown in synthetic medium with the addition of different amino acids. They found that the highest total cellular amino acid concentrations were found in yeast cells grown in the synthetic medium with the addition of histidine and lysine. An accumulation of glutamate was also observed, but mainly in the cytosol.

Amino acids released during on-lees aging do not all originate from the vacuole and cytosol compartments of yeast cells, but are also derived from the breakdown of polypeptides and proteins during hydrolysis in the presence of proteases. Martínez-Rodríguez and Polo (2000) studied amino acids released during autolysis of *S. bayanus* (EC1118) in a model wine. During the first four hours of aging, asparagine (Asn), glutamic acid (Glu), arginine (Arg), alanine (Ala) and ornithine (Orn) were the major amino acids released (Martínez-Rodríguez & Polo, 2000). These amino acids probably originated from the vacuole and cytosol through a process known as exsorption because self-proteolysis would not likely have begun. Exsorption is a phenomenon by which low molecular weight compounds undergo passive transfer to the extracellular environment (Feuillat & Charpentier, 1982; Martínez-Rodríguez & Polo, 2000). After 56 days of aging, the concentration of polypeptides and soluble proteins decreased from 90 (on the first day) to 36 mg/L (determined as bovine serum albumin equivalent), and total free amino acids increased from 237 to 316 mg/L, suggesting further protein hydrolysis took place during aging. The major amino acids released were alanine (Ala), γ-4-aminobutanoic acid, phenylalanine (Phe), and leucine (Leu) (Martínez-Rodríguez & Polo, 2000).

Peptides are also released during yeast autolysis. However, few studies have focussed on peptides in wine due to their complexity and the limitations of analytical techniques (Martínez-Rodríguez &

Pueyo, 2009). Moreno-Arribas et al. (1996, 1998a, 1998b) isolated and characterized peptides (into < 700 and > 700 Da fractions) generated during the production of sparkling wine using the traditional method. During yeast autolysis, larger peptides were hydrolyzed which gave rise to peptides with lower molecular weights and finally degraded to amino acids (Moreno-Arribas et al., 1996). Desporteds et al. (2001) identified low molecular weight peptides (< 3000 Da); in a further sensory study, four peptides (Ile-Val, Val-Ile, Phe-Arg-Arg, and Ser-Lys-Thr-Ser-Pro-Tyr) were described as bitter; and Phe-Lys and Lys-Met-Asn were found to be sour and umami, respectively. However, the concentration of these peptides were too low and below the sensorial threshold. The role of these peptides in wine flavour might be attributed to synergistic effects with other chemical components (Desporteds et al., 2001). Moreno-Arribas et al. (1998b) also studied the amino acid composition of isolated peptides in sparkling wine: serine (Ser) and threonine (Thr) were the major amino acids in peptides in both fractions. They concluded that the isolated peptides were mainly from the breakdown of yeast cell wall because Ser and Thr were components of the glycosidic bonds in mannoprotein of yeast cell walls.

2.3.2 Polysaccharides

Polysaccharides are the major component of yeast cell walls. In the cell walls of *S. cerevisiae* four distinct polysaccharides have been identified, mannoproteins, β-1,3 glucan, β-1,6 glucan and chitin, which account for 15-25% of the cell wall dry weight (Jiménez-Moreno and Ańcin-Azpilicueta, 2009; Feuillat, 2003).

2.3.2.1 Mannoproteins

Two differing mannoproteins are found in the yeast cell walls: the glycosylophophatidylinositol cell wall protein (GPI-CWP), and the cell wall protein with internal repeats (PIR-CWP).

GPI-CWP is believed to constitute the outer layer of the cell wall structure of *S. cerevisiae* (Klis et al., 2002). Mannose moieties are attached to proteins by the way of di-N-acetylchitobiose units linked to asparagine (Asp) through the amino group (Garrett & Grisham, 2010) (Figure 2.2). In a study of *S. cerevisiae*, GPI-CWP was found to be incorporated in the cell wall structure in different ways. For example, the GPI anchor of mannoprotein was attached to β-1,6 glucan, which in turn was covalently linked to β-1,3 glucan. This linkage was the most abundant one in cells grown in a rich medium (Klis et al., 2002).

PIR-CWP is a part of inner layer of cell wall structure and is covalently linked to β-1,3 glucan directly via an alkali-sensitive linkage.

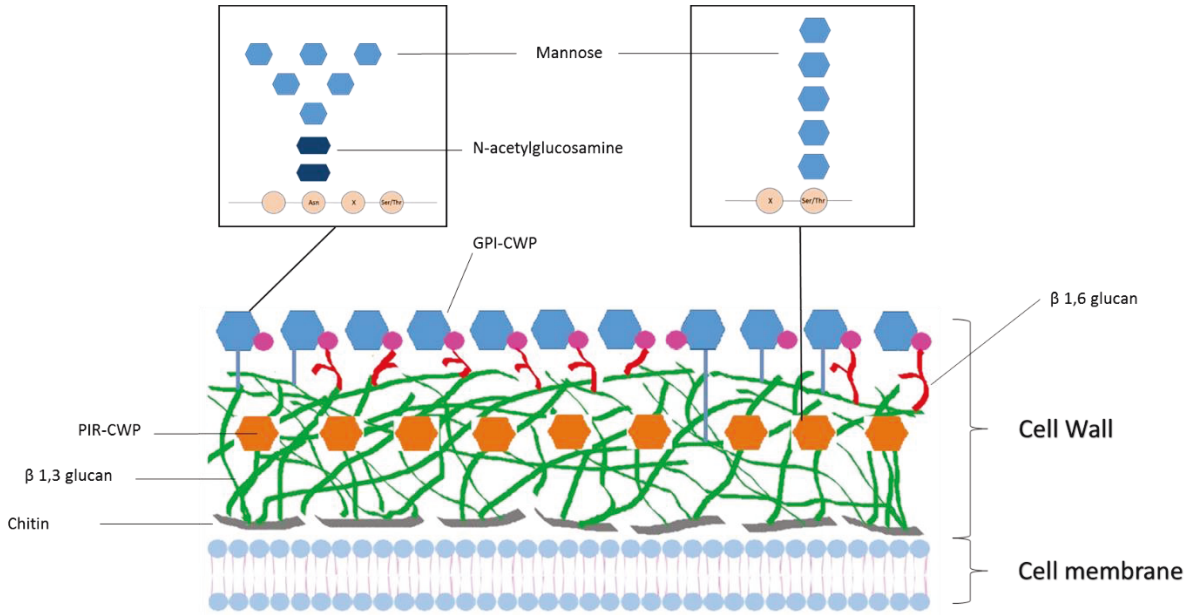


Figure 2.2 General structure of yeast cell wall.

2.3.2.2 Glucan and chitin

Two differing glucans (glucose polymers) are found in yeast cell walls: β-1,3 glucans and β-1,6 glucans (Douwes, 2005). A three-dimensional network of β-1,3 glucan forms an internal skeletal framework, which surrounds the entire cell to provide mechanical strength (Smits et al., 1999). β-1,3 glucans have c. 1500 monosaccharide units connected via glycosidic bonds. The average molecular weight of β-1,3 glucans is 240 kDa and they are insoluble in water (Luo et al., 2015; Smits et al., 1999; Lipke and Ovalle, 1998). Krainer et al. (1994) confirmed that β-1,3 glucans possessed a helical conformation by using solid state nuclear magnetic resonance. Furthermore, these helices can be a single helix, triple helix or random coil structures (Williams, 1997). In yeast cell wall, β-1,6-glucans are branches on β-1,3-glucan helices and interconnect cell wall proteins, glycosyl phosphatidylinositol and adjacent β-1,3-glucans (Figure 2.2) (Douwes, 2005; Smits et al., 1999). The β-1,6 glucans are water soluble polymers and comprise about 15% of the total glucans in yeast. They consist of 130 to 140 monosaccharide units connected via glycosidic bonds (Kocková-Kratochvílová, 1990; Lipke and Ovalle, 1998).

Chitin is a high-molecular weight linear polymer which is mainly located adjacent to the cell membrane and has a structural function to strengthen the skeletal framework (Ravi Kumar, 2000; Smits et al., 1999). Chitin consists of 2-acetamido-2-deoxy-β-D-glucose units linked through a β (1→4) bonds (Ravi Kumar, 2000). Chitin has a very similar chemical structure to cellulose except for the repeating units of N-acetyl-D-glucosamines (Garrett & Grisham, 2010). There are three different

arrangements of chitin: all-parallel chains, antiparallel chains, and pairs of parallel sheets separated by single antiparallel sheets. These are referred to as α-chitin, β-chitin and γ-chitin, respectively (Garrett & Grisham, 2010).

2.3.3 Glycogen and trehalose

Glycogen and trehalose are major intracellular saccharides that provide energy reserves to yeast cells (Kocková-Kratochvílová, 1990) and provide protection against ethanol (Lucero et al., 2000). Glycogen is consumed first during starvation with c. 50% used within a few minutes. Meanwhile, an energy conservation mechanism is activated and the production of trehalose increases 5-fold (Kocková-Kratochvílová, 1990). Trehalose is the major reserved energy source during prolonged starvation with about 90% degraded in 45 minutes (Kocková-Kratochvílová, 1990). Once all the reserved energy sources are exhausted, yeast cells start a self-degradation process called autolysis.

Glycogen is a branched glucose polymer. The general accepted primary structure of glycogen comprises glucose residues connected via α-1,4-glycosidic linkage. The secondary structure comprises two glucose chains linked to a third chain via α-1,6-glycosidic linkage. The aggregation of several secondary structures lead to the formation of rosette patterns (Roach et al., 2012).

Trehalose (α-D-glucopyranolsyl-α-D-glucopyranoside) is a disaccharide composed of two glucose units. Only some yeast species that produce the enzyme trehalase, such as *S. cerevisiae*, *S. carlsbergensis*, and *Torulopsis dattila*, are able to ferment (Kocková-Kratochvílová, 1990) and accumulate trehalose as a stress response when responding to heat, alcohol and osmotic shock (Roustan & Sablayrolles, 2004).

2.3.4 Nucleotides

Nucleotides, the basic building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are located in the nucleus of yeast cells. Nucleotides comprise a heterocyclic nitrogenous base, a pentose (e.g. ribose) and phosphoric acid. The nitrogenous bases of nucleotides are either pyrimidine or purine. There are three pyrimidine and two purines commonly found in cells which are cytosine (C), uracil (U) and thymine (T), and adenine (A) and guanine (G), respectively. The term nucleosides are used to describe a base and pentose linked via a glycosidic bond. In the DNA of *Saccharomyces cerevisiae*, guanine, adenine, cytosine and thymine constitute 18.3%, 31.7%, 17.4% and 32.6%, respectively, of the total composition (Kocková-Kratochvílová, 1990). In RNA, uracil takes the role of thymine.

2.4 Yeast autolysis

2.4.1 Processes

Once available extracellular nutrients are exhausted, proliferating yeast cells enter a stationary phase in which morphological, physiological, and biochemical changes occur to increase the resistance to environmental stress. These include thickening of the cell wall, accumulation of reserve carbohydrates and acquisition of thermotolerance (Werner-Washburne et al., 1993). During this period, the nitrogen compounds of the still living yeast cells including amino acids, polypeptides and proteins start to diffuse through the intact cell wall (Feuillat & Charpentier, 1982; Martínez-Rodríguez & Polo, 2000). The increase of the amino acids in the surrounding medium is accompanied by an increase in other compounds that could be cell wall peptides (Charpentier et al., 1986). Exsorption normally occurs within the first 24 hours. For example, a fast liberation of 1000 mg N/L from 10 g/L of rehydrated yeasts (*S. cerevisiae bayanus*) added to Champagne during 24 h incubation at 37°C (Feuillat & Charpentier, 1982) has been measured. Martínez-Rodríguez and Polo (2000) investigated the nitrogen compounds released during yeast autolysis in a model wine system and reported an exsorption phenomenon during the first 4 hours of an aging experiment; the concentration of amino acids, and polypeptides and soluble proteins were 68 mg/L and 6.1 mg BSA/L, respectively. In addition, amino acids released during the first 4 h represented c. 30% of the total released over a time period of 15 days.

Activation of proteases is generally considered to signal the start of yeast autolysis, and is indicated by a further sharp increase in the concentration of nitrogen compounds in the surrounding media. Protease A is the principal hydrolytic enzyme, and is released into the intracellular space due to the degradation of cell endo-structures. Alexandre et al. (2003) and Alexandre and Guilloux-Benatier (2006) demonstrated that protease A was responsible for 80% and 60% of the nitrogen release during autolysis in model wine and wine, respectively. However, proteases are initially inhibited by a specific cytoplasmic inhibitor until the degradation of the inhibitor which then triggers the activation of proteolysis (Alexandre and Guilloux-Benatier, 2006). This leads to the breakdown of intracelluar polymer components which accumulate in the space restricted by the cell wall. Other enzymes involved in yeast autolysis include a wide range of exo- and endo- β-1,3 glucanases (Matile et al., 1971; Adam, 2004). The activities of glucanases firstly releases mannoproteins from covalently linked glucans, after which the glucan moiety of mannoproteins is released. Finally, mannoproteins are hydrolyzed by proteolytic enzymes. The action of β-1,3 glucanase also creates pores in the cell wall which allows the release of intracellular components to the extracellular environment. Further autolytic degradation continues in the extracellular environment (Martínez and Pueyo, 2009).

Yeast autolysis can be induced physically (through the actions of temperature, osmotic pressure, freezing and thawing), chemically (pH, detergents, and antibiotics), and biologically (addition of enzymes, aeration and starvation). Addition of inactive and partially broken-down yeasts, as well as alteration of temperature, are commonly used techniques in wine research to accelerate the process of autolysis of yeast and improve the quality of wine (Charpentier and Feuillat, 1993). Todd et al. (2000) found that the K2 killer strains of *S. cerevisiae* can cause rapid cell death of sensitive strains and suggested that using a combination of K2 killer strains and sensitive yeast strains could accelerate the onset of yeast autolysis. The formation of pores in yeast cells can be achieved using technologies such as high pressure or pulsed electric field. The feasibility of applying a pulsed electric field (PEF) in winemaking was investigated by Liu et al (2006) and Puértolas et al (2010). PEF can be used to inactivate bacteria and yeast by rupturing the cell structure (Puértolas et al., 2010). However, the acceleration of yeast autolysis and the impacts on organoleptic quality of wine remains to be investigated.

2.4.2 Methods of on-lees aging

On-lees (*sur lies*) aging has been carried out under a wide range of conditions. The main factors that can be varied are lees type, aging temperature, and aging time. Different techniques are also applied during on-lees aging, with periodic stirring (so-called *battonage*) and enzyme addition (β-glucanase) being the most commonly used. In the wine industry, although precise aging conditions and methods are still highly dependent on the experience of the winemaker, there is a significant body of scientific literature on the effects of various methods applied during on-lees aging (Table 2.3). These are discussed in the following paragraphs.

Different lees materials used during on-lees aging include original fine lees produced during wine fermentation, rehydrated active yeasts and inactivated dried yeasts. As shown in Table 2.3, research studies using fine lees mainly contained dead yeast cells of *Saccharomyces cerevisiae*, and the reported amounts of lees used in wine aging were between 3 and 5% v/v. In other studies of yeast autolysis, active dried yeasts were rehydrated and added to model wines. In recent years, inactivated yeasts have been used to improve wine quality. These materials are obtained by thermal or enzymatic inactivation of viable yeast cells, and are often classified as yeast derivatives, yeast autolysates, yeast walls and yeast extracts (Pozo-Bayón et al., 2009). Commercial inactivated yeast products are designed by suppliers for different purposes including enrichment of polysaccharides, mannoproteins, nucleotides, and peptides (Del Barrio-Galán et al., 2011). Recommended dosage amounts vary between products and manufacturers. However, 40 g/hL of inactivated yeasts is the maximum authorized dosage according to the European Community (EC Regulation Nº 606/2009).

Table 2.3 indicates that temperatures used for on-lees aging normally ranged from 11 to 20 °C, and that periods of aging from 56 to 900 days have been used in scientific studies.

Periodic stirring is a conventional method used during on-lees aging. This method has been used in the aging of both white and red wines. In commercial practice, the rate and intensity of stirring are highly dependent on the experience of the winemaker. In research studies, wine stored on-lees is commonly stirred once or twice a week. Other vinification treatments have also been applied with periodic stirring, such as addition of sulphur dioxide and oxygenation. The time length of stirring was not specified in those studies except in a study that used a model wine where stirring was performed once a month for 10 min (Row et al., 2017). Gonzãlez Marco and Ancín-Azpiliceleta (2006) and Doco et al. (2003) found that stirring during aging increased specific amino acids (histamine and tyramine) and mannoprotein concentration, respectively, in the aged wine. However, the effect of periodic stirring on the release of chemical compounds from yeast cells is rarely reported.

Commercial enzyme products have been developed for the acceleration of yeast autolysis in wines aged on-lees. These are often a mix of different enzymes, such as β-glucanse and pectinase (Palomero et al., 2007). Currently, β-glucanse synthesised by *Trichoderma* sp. is the only authorised enzyme for use in winemaking (Humbert-Goffard et al., 2004). The dosage of β-glucanse used in previous studies ranged between 10 and 60 mg/L. Rodriguez-Nogales et al. (2012) found that β-glucanse seem to increase the aging characteristics of the sparkling wine. β-Glucanse was also found to increase the polysaccharide content of wines. For example, polysaccharide content was increased from 3 to 57 mg pullulan equivalent/L in the model wine in the presence of 50 mg/L of Lallzy MMX (Palomero et al., 2007). In addition, it can also influence the free amino acids profile in sparkling wine by increasing the release of amino acids (Torresi et al., 2014).

Periodic stirring and addition of β-glucanse can be both applied during on-lees aging. For example, Bombino bianco wine was stirred daily for 70 days in the presence of β-glucanse (30 and 60 mg/L); the content of hexanol and trans-3-hexenol were strongly increased by the presence of lees and by enzymatic treatments (Masino et al., 2008). In general, periodic stirring and enzyme addition are able to increase the release of chemical compounds from yeast cells. However, the effect of stirring rate and intensity and their interaction with enzyme addition are still poorly investigated.

2.4.2.1 Pulsed Electric Field

During the last decade, pulsed electric field (PEF) technology has attracted the attention of a number of researchers. Studies have mainly focused on the enhancement of extraction, the induction of yeast autolysis and the acceleration of maturation (Table 2.4). PEF is a novel, non-thermal processing

technique which delivers microsecond high voltage pulses to a material placed between two electrodes (Puértolas et al., 2010). This technology is now widely used in the food industry to treat foods and beverages that have low electrical conductivity and do not contain or form bubbles (Elez-Martínez et al., 2012). Studies of application of PEF on winemaking can be traced back to early 2000s. All three applications above are potentially able to improve cost efficiency of winemaking. For example, both induced autolysis and accelerating maturation are able to reduce the cost of labour and storage, and enhancement of extraction is able to increase profit by increasing the yield of juice. In addition, the obtained juice was found had a higher content of total phenolic and vitamin C which improved its bioactivity and quality on health aspects (Leong et al., 2016).

The concept PEF was first proposed in 1967 to change the behaviour of microorganisms (Ravishankar et al., 2008). Castro et al. (1993) demonstrated the mechanism of PEF for microbial inactivation (Figure 2.3). In a pulsed electric field, opposite charges at the membrane surfaces attract each other. Once the electrical field strength reaches a certain threshold level, the electric compressive forces cause local breakdown of the membrane and lead to the formation of a pore (Castro et al., 1993). The type of microorganism, pH, treatment time and field strength are four possible factors determine the degree of the damage inflicted on the cell membrane (Elez-Martínez et al., 2012).

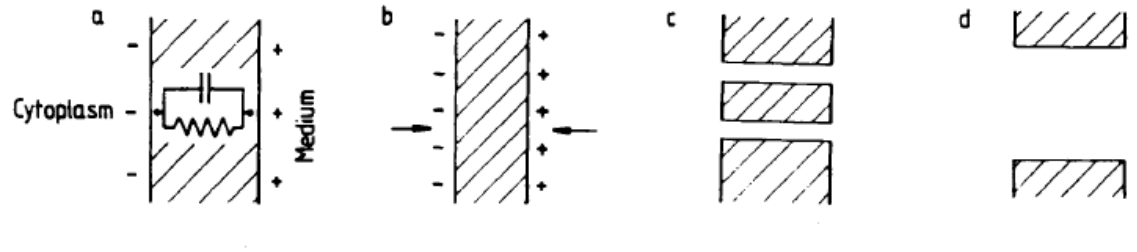


Figure 2.3 Mechanism of pore formation in a cell wall by PEF.

a. Free charges accumulated at both membrane surfaces; b. PEF induced compression; c. Pore formation; d. Large pore formation. Source (Castro et al., 1993)

Table 2.3 Summary of conventional methods used in on-lees aging in experiments conducted in model and white wines.

Wine	Yeast	Lees type	temperature	Agitation	Enzyme	Aging period	Effects of conventional aging technique	Reference
Model wine	S.cerevisiae	original lees	15 °C	stirring once a month for 10 mins	N/A	285 days	N/A	Rowe et al., 2017
Model wine	S.cerevisiae	rehydrated yeasts	18 °C / 30°C	No stirring	N/A	56/36 days	composition depends on growth medium and physiology state of yeasts	Guilloux-Benatier & Chassagne, 2003
Model wine	S.cerevisiae	Lyophilised yeasts	30°C	weekly orbital agitation for 1 hour	Lallzy MMX 50 mg/L	270 days	β-glucanase accelerated the process of autolysis. Polysaccharide increased from 2.70 to 57.20 mg pullulan equivalent/L in 3 weeks	Palomero et al., 2007
Chardonnay	N/A	N/A	N/A	weekly stirring with 12 ppm sulphur addition	N/A	180 days	weekly stirring influnce histamine and tyramine concentration. Higher than beginning of aging	González Marco & Ancín-Azpilicueta, 2006
Chardonnay	S.cerevisiae	original fine lees	N/A	stirring twice a week	N/A	300 days	N/A	Liberatore et al., 2000
Chardonnay	S.cerevisiae	original fine lees	N/A	stirring twice a week	N/A	300 days	N/A	Pati et al., 2010
Chardonnay	S.cerevisiae	original fine lees	N/A	stirring once a week	N/A	180 days	N/A	Ancín-Azpilicueta et al., 2009
Asytiko, Chardonnay	N/A	original fine lees	N/A	N/A	N/A	197 days	N/A	
Sparkling	S. cerevisiae EC1118 & BCS103	original fine lees	14 °C	N/A	Lallzy MMX 10 mg/L	365 days	β-glucanase improved the release of amino acids into the wine; influence free amino aicd profile with yeast dependent effects	Torresi et al., 2014
Sparkling	S. cerevisiae var. bayanus	original lees; yeast products (15-30 mg/L)	11-13 °C	no stirring	N/A	270 days	N/A	Pérez-Magariño et al., 2015
Madiran	N/A	original lees (5%)	N/A	stirring weekly with/without oxygenation	N/A	180 days	stirring of lees during the aging allowed a slight increase of mannoprotein	Doco et al., 2003
Bombino bianco	Lalvin ICV-D47	N/A	18-20 °C	daily sitrring by keg overturning	β-glucanase 30-60 mg/L	70 days	enhanced all volatile compounds	Masino et al., 2009
Verdejo	S.cerevisiae	original fine lees	15 °C	stirring every 3 days	2000 mg/L	60 days	N/A	Del Barrio-Galan et al., 2011
Verdejo	Uvform CS2	original fine lees	N/A	stirring once a week	N/A	180 days	N/A	Losada et al., 2012
Verdejo sparkling	S. cerevisiae var. bayanus	original fine lees; yeast derivatives	14-15 °C	N/A	β-glucanase 10-30 mg/L (428 UI/g)	270 days	β-glucanase seem to increase the aging characteristics of the sparkling wines, while derivatives improve fruity and flowery character	Rodriguez-Nogales et al., 2012
Tempranillo/Verdejo	N/A	original fine lees	16 °C	twice a week	N/A	56 days	N/A	Del Barrio-Galan et al, 2012
Glalician blend	N/A	original fine lees	N/A	N/A	N/A	60-210 days	N/A	Bautista et al., 2007

N/A: information not available in the cited reference

Table 2.4 Summary of application of PEF in winemaking.

Basic information			Treatment conditions					Effects	Reference
Variety	Brix	Sulphur (ppm)	No. of Pulses	Strength (kV/cm)	Frequency (Hz)	Stage applied	Energy Consumption (kJ/kg)		
Parellada	-	-	-	35	303	Pre-fermentation	1500,000kJ/kg	Yeasts and bacteria were inactivated with reduction rangede from 2.24-3.94	Marsellés-Fontanet et al 2009
-	-	-	0-100	16-31	1	red wine/must	10-350	The optimum treatment of 186kJ/kg at 29 kV/cm were able to reduce 99.9% of the spoilage flora in must and wine.	Puértolas et al, 2009
Parellada	-	-	-	35	1000	Pre-fermentation	-	PEF was used for sterilization of must; The consumption of amino acids was higher when SO_2 present during fermentation	Garde-Cerdán et al, 2007
Cabernet Sauvignon	25.8	30	50	5	122	Maceration/fermentation	3.67	The total concentration of flavan-3-ols, flavonols and phenolic acids were increased 20%, 37% and 25%. PEF is more effective than enzymatic preparation	Puértolas et al, 2009
Cabernet Sauvignon	-	30	50	5	1	Pre-Maceration	2.1	The total polyphenol index was 22.7-45.2% higher than control wine; Maceration time of PEF treated pomace showed no important influcen on yellow colour.	López et al, 2009
Cabernet Sauvignon	23.5	30	50	5	122	Pre-Maceration	3.67	Better chromatic characteristics and higher phenolic content; No sensory differences in colour and bouquet.	Puértolas et al, 2010
Cabernet Sauvignon	23.5	30	50	5	122	Pre-fermentation	3.67	No differences in the content of monimeric anthocyanin; higher flavan-3-ols, flavonols and hydroxycinnamic acids and derivatives in PEFed wine.	Puértolas et al, 2010
Cabernent Sauvignon, Rosé	24.8	60	50	5	122	Pre-fermentation	3.67	higher anthocyanin content after 2 months aging in bottle; Reduce maceration time	Puértolas et al, 2011
Tempranillo	25.8	-	50	5, 10	1	Pre-fermentation, PEF treated skin only	1.8, 6.7	For E=5 and 10 kV/cm, no effect on the alcohol content, pH, reducing sugars, volatile acidity and total acidity. Both increase total phenol content.	López et al, 2008

Table 2.4 (continued)

Basic information			Treatment conditions					Effects	Reference
Variety	Brix	Sulphur (ppm)	No. of Pulses	Strength (kV/cm)	Frequency (Hz)	Stage applied	Energy Consumption (kJ/kg)		
Dornfelder	-	-	30	3	2	By-product	-	Total phenolic content incrased two-fold; Antioxidant increased four-fold	Corrales et al, 2008
Garnacha, Graciano, Mazuelo	23.0-27.9	30	50	2, 5 and 10	1	Maceration	0.4, 1.8 and 6.7	Improve the extraction of phenolic compounds; Effectiveness of PEF on colour, anthocyanin and index of polyphenol depended on grape variety.	López et al, 2008
Muscadelle, Sauvignon, Semillon	22.4,21.4, 22.5	-	100	0.75	-	Pre-pressing	20	PEF enhanced expression is promising for production of higher quality juices in the wine indudstry	Praporsic et al, 2007
Chardonnay	-	-	2	0.75	-	-	20	PEF improved the extraction of phenolic compounds	Boussetta et al, 2009
Chardonnay	-	-	2	0.4	-	Beginning of pressing	15	Juice yield increased 67%-75%; Increase total phenol content >15% for the progressive pressure-increase regime.	Grimi et al, 2009
Cabernet Sauvignon	-	-	-	0.6	3000	Post-MLF, no lees	-	The optimum treatment is 0.6kV/cm for 3 mins. Higher alcohol and aldehydes decreased, esters and free amino acid increased slightly.	Zeng et al, 2008
Aglianico, Piedirosso, Nebbiolo, Casavecchia	-	5	-	1.5, 3.0	1, 10	Pre-Maceration	10, 20	Aglianico grapes: significant higher polyphenol, anthocyanins content and colour intensity than control. Other three had minor increase.	Donsì et al, 2011
Dunkelfelder	-	50	-	1.2	1000	Wastes	18	Densification and PEF are efficient combination. Increased the content of total phenols regardless of time. Also higher in anthocyanin and flavanol-3-ols.	Briancean et al, 2014
Graciano, Tempranillo, Grenache	-	70	-	7.4	300, 400	after pressing	-	PEF enhanced aromatic composition of Grenache; increase monoterpenoids,β-ionone, total esters and benzenoid. No effects on Tempranillo and Graciano.	Garde-Cerdán et al, 2013

Table 2.4 (continued)

Basic information			Treatment conditions					Effects	Reference
Variety	Brix	Sulphur (ppm)	No. of Pulses	Strength (kV/cm)	Frequency (Hz)	Stage applied	Energy Consumption (kJ/kg)		
Model wine	-	-	-	0, 30, 40, 50	1080	-	-	PEF enhanced the condensation reaction between catechin and acetaldehyde. Temperature and pH are also the parameter affect the reaction.	Zhao et al, 2013
Cabernet Sauvignon	22	-	100, 1	0.7, 4	100	after crushing	14.4, 111.6	Long duration and 4 kV/cm changed the structure of grape skin, and improve tannin extraction 31%; 0.75 kV/cm increase extraction of anthocyanin by 19%.	Delsart et al, 2014
Merlot	-	20	200	0.5-0.7	90	pre-crushing	-	Increased extraction of polyphenol and anthocyanins; PEF contributes to the enhancement of the senosry attribute of wine	Delsart et al, 2012
Graciano, Tempranillo, Grenache		70	-	7.4	300, 400	post-crushing	-	Increased potassium content, CI, total polyphenol and lowered tonality. Increase depended on the variety and the treatment applied	López-Alfaro et al, 2013
Cabernet Franc, Cabernet Sauvignon	26.7-27.7, 25.7-26.1	-	100	0.4, 0.8, 5	-	post-crushing	3.1-48.0	5kV/cm, 1ms, 48kJ/kg enchanced quercetin 3-β-D-glucoside and epicatechin gallate, CI and anthocyanin. Low treatment less effective on phenol extraction	Darra et al, 2013
Garnacha	25.3	-	20	4	250	post-crushing	1.5	Increase of CI, anthocyanin and TPC were found after 7 days maceration. No difference with control after 14 days maceration. Sensory analysis showed PEF treated wine is more preferable than control.	Luengo et al, 2014
Cabernet Sauvignon	-	-	-	0.6	3000	post MLF	-	Wine aging accelerated, volatile higher alcohol and aldehyde decreased; the content of estersand free amino acids sligthly increased.	Zeng et al, 2008
Cabernet Sauvignon	-	-	-	0-10	1000	post MLF	-	The hydrogen bond between ethanol and water molecule increased while the taste became softer;	Xiao et al, 2004
Cabernet Sauvignon	-	-	-	0-5	1000	post MLF	-	The content of fusel oil (isoamyl alcohol) were decreased while the total ester were increased. Wine softened and full bodied.	Zeng et al, 2004
Red wine	-	-	10	6.5-35	3000	post MLF	-	Increased colour stability, reduce alcohol level. It can be used to accelerate wine maturation	Yin et al, 2006
Red wine	-	-	10	0-20	3000	post MLF	-	Reduced fusel oil content; Increased aging aroma, enjoyable flavour and more full-bodied.	Liu et al, 2006
Cabernet Sauvignon	-	-	-	2, 5	-	post MLF	-	Higher content of free amino acids in treated samples. The wine body is more enjoyable.	Chen et al, 2004

There have been many studies focused on the application of PEF to microbial inactivation of foods including beverages. For example, PEF was used to effectively inactivate *Escherichia coli* in different types of juices and skim milk using various treatment parameters (Akın & Evrendilek, 2009; Evrendilek et al., 2000; McNamee et al., 2010; Mosqueda-Melgar et al., 2007). There have been fewer studies of the application of PEF to microbial inactivation in grape juice and wine (Table 2.4). Nevertheless, PEF has been demonstrated to effectively inactivate bacteria and yeasts in juice and wine (Álvarez et al., 2006; Marsellés-Fontanet et al., 2009).

In a different context, Garde-Cerdán et al. (2008) demonstrated that after PEF treatment of must, the addition of sulphur dioxide can be reduced. Sulphur dioxide addition is a common practice in winemaking to inhibit undesirable microorganisms and reduce and/or prevent oxidation, but at high concentrations it has a negative impact on wine flavour. It inactivates certain grape enzymes to prevent loss of quality of juice. However, the impact of the combination of addition of sulphur dioxide and PEF on the flavour of the specific wine needs to be further investigated.

Pulsed electric field can be used for the induction of yeast autolysis. This can be achieved at low and high PEF intensity. Ganeva et al. (2014) treated yeast cells with a relatively low PEF intensity (2.5 to 4.5 kV/cm for 0.1 to 2 ms); cytoplasmic proteins were released without yeast cells lysis. In addition, the release of protein from low PEF intensity treated yeasts can be enhanced in the presence of a low dosage of lyticase. For example, the addition of lyticase (1 U/mL) enhanced release rate of protein without causing cell lysis (Ganeva et al., 2014). At high PEF intensity, yeast cells can be damaged and mannoproteins are released from yeast cell walls. Martínez et al. (2016) monitored the release of mannoprotein and intracellular material from PEF induced yeast cells (5 -25 kV/cm, 30 – 240 μs) for 18 days, and found the amount of mannoprotein released was 4.2 times higher than the untreated yeasts. Several factors are able to affect the release of the chemical compounds during PEF-induced autolysis including time, temperature, pH and ethanol concentration (Ganeva et al., 2014; Martínez et al., 2016; Martínez et al., 2018). In a short incubation of PEF induced yeast cells, Ganeva et al. (2014) and Martínez et al. (2016) demonstrated the increases of protein and mannoprotein concentrations, and achieved the maximum after 8 and 432 hours, respectively. Martínez et al. (2018) investigated the influence of temperature (7 -43°C), pH (3.5 -7.0) and ethanol concentration (6 -25%) on PEF induced autolysis of *Saccharomyces cerevisiae*. The results showed that the concentration of mannose in the media achieved 90 mg/L after 21 days of incubation at 43°C; however, the release of mannose was slower under normal wine conditions (10% v/v ethanol, at pH 3.5) (Martínez et al., 2017). The release of different compounds from PEF induced on-lees aging remains poorly investigated. Further work needs to be done to investigate the effect of PEF on

different lees material (or yeast strain), interactions with conventional vinification techniques (e.g. stirring and enzyme addition) and benefits to wine quality.

2.5 Release of compounds during yeast autolysis and on-lees aging

2.5.1 Nitrogenous compounds

Nitrogenous compounds released during yeast autolysis consist of proteins, polypeptides, peptides and amino acids (Fornairon-Bonnefond et al., 2002; Martínez-Rodríguez and Polo, 2000). During yeast autolysis, an increase of the total nitrogen content is expected due to the autolytic release of nitrogenous compounds from yeasts (Martínez-Rodríguez et al., 2001; Feuillat and Charpentier, 1982). Ferrari and Feuillat (1988) demonstrated that wine aged on lees had a higher nitrogen and amino acid content. They also determined total nitrogen and total amino acids to be 40% greater in the wine with periodic stirring than no stirring. Leroy et al (1990) and Martínez-Rodríguez and Carmen Polo (2000) monitored yeast autolysis during on-lees aging by determining total nitrogen using the Kjeldahl method. In a 15-day autolysis experiment with rehydrated dry yeasts (10 g/L), the total nitrogen content increased from less than 10 mg/L to 234 mg/L within 20 h and then continued to increase gradually to 332 mg/L over the following 6 days

2.5.1.1 Protein

Effect of aging time

In general, a significant increase of protein concentration is expected immediately following exsorption; this is attributed to the release of the accumulated proteins and intracellular proteases through the damaged yeast cell wall. However, the amount of the accumulated proteins and proteases remained unknown. Following this, with the presence of protease in the extracelluar medium, the changes of protein content will depend on the rate of release of macromolecules and hydrolysis of the released protein. For example, the protein concentration will decrease if the rate of hydrolysis of the released protein is lower than the release of protein and mannoprotein, and *vice versa*. Eventually, the protein content will decrease since the majority of proteins will be broken down to peptides and amino acids.

The release of protein during yeast autolysis was investigated in previous studies using different yeast materials, e.g. rehydrated active yeasts, harvested fresh yeasts, inactivated yeasts, and the collected lees (yeast lees) from wine production. Martínez-Rodríguez and Polo (2000) measured nitrogen compounds released from rehydrated yeasts (*Saccharomyces bayanus,* EC1118) during yeast autolysis in a model wine over a period of 15 days. The concentration was determined by

Bradford method with the results expressed as bovine serum albumin (BSA) equivalent (Bradford, 1976). The concentration of soluble protein and polypeptides (with a molecular weight greater than 10 kDa) increased from 6 mg BSA/L to 55 mg BSA/L in the first 24 hours, followed by a gradual decrease to 32 mg BSA mg/L at the end of the aging experiment as the result of degradation of proteins and polypeptides to amino acids.

Juega et al (2015) carried out a short aging on-lees experiment in Albariño wine. Base wines were separated into five portions and then were aged with lees for 10, 20, 30, 40, and 50 days. The results showed no significant differences in protein concentration between the wines that had aging times of no more than 20 days. The highest protein concentration was found in the wine aged for 30 days (60.3 mg/L) which suggested the beginning of the yeast autolysis. The least protein concentration was found in the wine aged for 50 days (18.8 mg/L) which can be attributed to the breakdown of protein in the presence of free protease; small peptides and amino acids (molecular weight < 10 kDa) were not able to be detected by Bradford protein analysis used in this study (Juega et al., 2015).

A similar finding was also reported by Martínez-Rodríguez and Polo (2000). In a 6-month aging trial with fresh lees (3% w/v of fine lees) and three different dry yeast derivative products (doses: 0.4 g/L, 0.4 g/L and 0.05 g/L) in white wine (with *batonnage* twice a week for 60 days before aging in bottle), Del Barrio-Galán et al (2011) reported a decrease in protein concentration from the end of fermentation (66.0 mg BSA equivalent/L) to the end of 6 months (< 5.0 mg BSA equivalent/L).

Effect of pH

Zhao and Fleet (2003) investigated changes in protein concentration at four pH levels (4.0, 5.0, 6.0 and 7.0) using harvested fresh yeasts (*Saccharomyces cerevisiae* strain x280a). For autolysis at 40°C, they found that the rate of increase was greater at pH 5 and pH 7 (130-140 mg/L at day 10) compared to that at pH 4 (87 mg/L at day 10). Fornairon-Bonnefond and Salmon (2003) applied constant stirring (500 rpm at 28°C; pH 3.3) in the presence of argon or oxygen to synthetic wine, and the protein released from harvested yeasts to synthetic model wine was ranged from 67.5 to 85.5 mg/L at day 15. Even though the stirring in the presence of oxygen showed a more rapid release of protein than the one with argon, no differences were detected in final protein concentration in the media at the end of aging experiment (after 21 days).

Previous studies have demonstrated common methods that can be used for monitoring the protein concentration during yeast autolysis; and that aging time, pH, type of lees and periodic stirring were the factors that affect protein concentration in model medium or wine. However, the effects of aging time, type of lees, stirring frequency and addition of β-glucanases and their interactions on

protein concentration during yeast autolysis are poorly investigated.

2.5.1.2 Amino acids

Effect of lees types

Low concentrations of amino acids are expected in the early stage of yeast autolysis. Then, in general, amino acid content in the medium is expected to continuously increase throughout yeast autolysis due to the hydrolysis of proteins and mannoproteins (Fornairon-Bonnefond et al., 2002). The increase is expected to slow down and eventually cease as the content of protein and mannoprotein declines; the concentration of amino acids may also decrease towards to the end of yeast autolysis. Thus, Martínez-Rodríguez et al. (2001) also demonstrated free amino nitrogen content released by three different rehydrated yeasts (*Saccharomyces bayanus*, EC1118, BC, and PM) during autolysis in model wine all increased sharply during the first 24 h to three different levels, and remained constant for the rest of aging period. This suggested that the concentration of most free amino acids was significantly affected by the strain. The decrease of the concentration of amino acids towards to the end of yeast autolysis had been explained by Suárez et al. (2005) as deamination reactions of amino acids or participation in the formation of different compounds.

In terms of the amino acid profile, Alexandre et al (2001) reported that the amino acids Lys, Val, Ala, Pro, Gln, Gly, and Glu represent about 75% of the total in the medium during alcoholic fermentation; during autolysis, other amino acids such as Asp, Arg, His, Leu, Thr, and Ser start to appear. Van Der Vaart et al (1995) suggested that the appearance of Thr and Ser was evidence of the degradation of mannoprotein because these two amino acids are the major ones in the C-terminal region of the yeast cell wall. Temperature was demonstrated to influence amino acid content. At the end of a 6-month on-lees aging trial, the amino acid content (not including proline) of Kushu wine stored at 20°C was found about 40 mg/L higher than the one stored at 10°C (Sato et al., 1997). Previous studies explained the changes in the concentration of amino acids during the yeast autolysis and pointed out the correlation between yeast autolysis and specific amino acids (e.g. Thr, Ser and Asp). However, the effects of aging time, type of lees, stirring frequency and addition of β-glucanases and their interactions on total free amino acids in model medium and wine are remained poorly investigated. In addition, the changes in Asp, Arg, His, Leu, Thr, and Ser contents during yeasts autolysis need to be investigated in further studies.

2.5.2 Polysaccharides

Both neutral (the majority) and acidic polysaccharides are released during yeast autolysis, so that the total polysaccharide content of wine increases with aging time. This has been observed in

studies with red, white and model wines (Del Barrio-Galán et al., 2014; Del Barrio-Galán et al., 2015; Palomero et al., 2009; Pati et al., 2010). The increase in the total polysaccharide concentration during yeast autolysis can be attributed to the release of low-molecular weight polysaccharides. Del Barrio-Galán et al. (2014) and Del Barrio-Galán et al. (2015) quantified different polysaccharide molecular weight fractions weight (i.e. > 2000 kDa, 200 – 300 kDa, 60 – 80 kDa, and ≥10 but ≤60 KDa;) during red wine aging. The highest polysaccharide concentration was found for the 60-80 kDa and ≥ 10 but ≤60 kDa fractions. Following an initial increase, some studies have shown a decrease in total and neutral polysaccharide content. This can be attributed to a change of solubility. Pati et al. (2010) suggested that active hydrolytic enzymes are able to reduce polysaccharide molecular weight, change their composition, and modify their hydrophobic properties and consequently their solubility.

Different techniques used for acceleration of wine aging (selection of yeast strains, use of yeast derivatives, and addition of enzymes) have been shown to affect polysaccharide concentration during yeast autolysis.

Effect of yeast strains

Palomero et al. (2009) used different yeast strains (*S. cerevisiae* G3, *Schiz. pombe* 936, *Saccharomycedes ludwigii* 980, *Pichia anomala* 930, *Pichia membranifaciens* 956) in a model wine for 142 days, and found that the greatest amount of polysaccharides was released from *Saccharomycodes ludwigii* 980 (110.51 mg/L of pullulans) and the least amount from *Pichia membranifaciens* 956 (21.2 mg/L of pullulans). The differences were likely to be due to the differences in the chemical composition and structure of yeast cell wall (Weijman & Golubev, 1987).

Effect of addition of yeast derivatives

Yeast derivatives are sometimes added to wine to increase polysaccharide content (Del Barrio-Galán et al., 2011, 2012; 2014). Del Barrio-Galán et al. (2012) evaluated several yeast derivative products [obtained from: Agrovin (autolyzed yeast enriched in polysaccharides and/or β-glucanase activity), Sepsa (polysaccharides from selected yeast cell walls), Laffort (contains peptide fractions), Bio Springer (high in polysaccharide and mannoprotein) and AEB (rich in mannoprotein and nucleotides)] rich in polysaccharides and reported higher total and neutral polysaccharide concentrations than control after 8 weeks of on-lees aging treatment and 3 months of bottle aging. The results suggest that the addition of yeast derivative products does not produce an immediate release of the polysaccharides and that this release continues during wine aging. In addition, the increase of polysaccharide was dependent on the type and purity of yeast derivatives used (Del Barrio-Galán et al., 2011).

Effect of enzyme addition

The addition of the enzyme β-glucanase has been shown to accelerate yeast autolysis (Palomero, et al., 2007; Torresi, et al., 2014). Even though β-glucanase can be inhibited by ethanol, its residual activity is sufficient at wine pH to allow more rapid breakdown of yeast cells wall compared to conventional autolysis (Torresi et al., 2014). Palomero et al. (2007) evaluated the effect of the addition of β-glucanase on the amount of polysaccharide during yeast autolysis in the model medium and reported that in just two weeks the polysaccharide content (25.6 – 68.8 mg pullulan/L) was similar or higher than the ones produced in nine months (21.8 – 36.8 mg pullulan/L) by conventional methods. In addition, the addition of β-glucanase is able to produce smaller polysaccharide fragments with more uniform in size. For example, the molecular weight of polysaccharides released from *Saccharomyces cerevisiae* strain 9CV in the presence of β-glucanases was approximately 35 kDa; in contrast, the molecular weight of polysaccharides released by conventional methods was approximately 120 kDa.

2.5.3 Mannoprotein and glucan

It is difficult to measure mannoprotein and glucan in wine directly. Therefore, the content of mannoprotein and glucan during yeast autolysis were usually represented using the concentrations of mannose and glucose after hydrolysis of these two compounds, respectively (Dupin et al., 2000; Salmon et al., 2003; Martínez-Lapuente et al., 2013). In general, the content of mannoprotein is low at the beginning of yeast autolysis and expected to increase as autolysis progresses. Dupin et al. (2000) evaluated the changes of mannoprotein content during yeast autolysis in the model wine, and its content progressively increased from 0 to 15 mg/L after eight weeks. This is in agreement with the finding reported by Salmon et al. (2003), in which mannoprotein increased from 0 to about 50 mg/L after aging of 12 months. In more detail, mannoprotein may be released to the medium earlier than glucan. This was attributed to the cell wall structure in which mannoprotein is covalently linked to the glucans and it is first released by endo- and exo-β-(1,3)-glucanases, and then glucans (Martínez-Lapuente et al., 2013). In addition, the release of mannoprotein also led to an increase in neutral polysaccharide in the medium; thus, the addition of β-glucanases should show a higher content of neutral polysaccharide (Rodriguez-Nogales et al., 2012).

The content of mannoprotein may gradually decrease towards the end of autolysis. Martínez-Lapuente et al. (2013) demonstrated a decrease of mannoprotein over an aging period longer than six months in sparkling wine. This was due to the aging conditions utilized (low pH and temperature, and high pressure of CO_2), reduction of hydrolytic enzymes activities due to lack of stirring and rate

of mannoprotein release and solubilization. A decrease of mannoprotein also can be attributed to its break-down under the synergistic effects of proteases and β-glucanase. Rodrigues et al. (2012) demonstrated that the protein and glucan moieties of mannoprotein can be hydrolyzed by proteases and β-glucanase, which led to the release of low molecule weight peptides and peptidemannans, respectively.

The mannose/glucose ratio also varies during yeast autolysis. Martínez-Lapuente et al. (2013) demonstrated an increase mannose/glucose ratio during in sparkling wine aging from 18 to 30 months. This was attributed to the reduction of glucose content during yeast autolysis and indicated that glucan might form more unstable compounds more susceptible to precipitation than mannoproteins. The mannose/glucose ratio is generally more stable towards the end of yeast autolysis (Charpentier et al., 2004)

2.6 Sensory effects of on-lees aging

2.6.1 Mouthfeel

The mouthfeel benefits of the 'aging on lees' technique on wine have been described in many studies (Table 2.3) (Escot et al., 2001; Del Barrio-Galán et al., 2011; Fernández et al., 2011; Wang, 2014; Del Barrio-Galán et al., 2011). However, there is still no commonly accepted definition for the term 'mouthfeel'. In general, mouthfeel is considered as the combination of sensations characterized by a tactile response in the mouth. In wine, the common in-mouth perceptions comprise astringency, body, touch, viscosity, burning, pain, temperature and prickling, and are responses to the major chemical constituents including phenolics, sugar, acid, and alcohol (Jackson, 2009).

Phenolic compounds play an important role in the mouthfeel of red wine due to their contribution to astringency. For example, the intensity of astringency was found to be correlated with increasing chain length of tannins, with words describing the different types of astringency such as 'drying', 'chalky', 'adhesive' and 'pucker' (Vidal, 2003). According to Pickering and Demiglio (2008) "the polyphenolic constituents of white wine also elicit astringent sensations although their concentration is significantly lower than in red wine".

Mannoproteins have been found to be compounds that are responsible for reducing astringency during aging red wine on lees (Escot et al., 2001; Del Barrio-Galán et al., 2014; Fernández et al., 2011). Vasserot et al. (1997) and Mazauric and Salmon (2006) studied the chemical composition of polyphenolic compounds (anthocyanins and condensed tannins) in lees and model solutions and

showed that the mechanism of reduced astringency was attributed to the adsorption of polyphenolics by surfaces of lees (mannoproteins). For example, mannoproteins isolated during alcoholic fermentation and autolysis were added back to the red wine by Escot et al. (2001) and the wines were found to be less astringent. Del Barrio-Galán et al. (2011) studied aging Tempranillo wine on lees (3% v/v fine lees) for 6 months in two vintages, and reduced astringency and green tannins were found compared to wines without on-lees aging. In addition, mannoproteins were found able to reduce haze formation, prevent precipitation of tartaric acid, improve the foam quality of sparkling wine, and improve the mouthfeel of wine (Dupin et al., 2000; Moine-Ledoux et al., 1997; Brissonnet & Maujean, 1991; Arribas et al., 2000; Alexandre & Guilloux-Benatier, 2006).

The investigation of the sensorial effects of using lysated lees and yeast derivatives during aging has attracted the attention of researchers. Del Barrio-Galán et al. (2011) achieved better mouth feel in wine by replacing lees with yeast derivatives (Super Bouquet, Agrovin, Spain). Moreover, Fernández et al. (2011) treated lees (recovered post-malolactic fermentation) with tartaric acid (2.5 g/L) and sulphurous acid to 40 mg/L of free SO_2, then added lysated lees back to wine with and without commercial enzymes (15 g/hL, mixture of pectinases and β-glucanses). The results clearly showed that lysated lees had a major effect on the mouthfeel, enhancing acid and fresh sensations; furthermore, acidification plus enzyme treatment increased sweetness, fullness, and mouth-length perception.

Ribeiro et al. (2014) studied the effects of commercial mannoproteins on white wine protein stabilization, and chemical and sensory properties. The results showed that body, balance, and persistence were related to the protein content of mannoprotein. Application of lees treated with microwave and heat, yeast derivatives and inactivated dry yeasts were studied by Wang (2014) to improve wine sensory quality and to reduce aging time. Lees were treated by heat (85°C) and microwaves (300 W). The results showed that the use of microwaved lees resulted in only ‘a little’ sensorial improvement in wine body; both treatments showed negative impacts on creaminess and length which normally increases in traditional aging on lees. Wang (2014) also replaced lees with commercial inactivated dry yeast in making Chardonnay which improved wine mouthfeel by reducing astringency and increasing length and body. Both lees and three different yeast derivatives were used in the aging of Verdejo white wine, which showed that all treated wine, especially the ones treated with yeast derivatives had higher scores for mouthfeel and overall rating and lower scores for acidity and astringency in comparison with the control wine; no statistical differences were detected between the wines treated with different yeast derivatives (Del Barrio-Galán et al., 2011).

Table 2.3 Summary of sensorial effects of aging on lees technique.

Grape variety	Materials used in aging	Sensory effects	Reference
Pinot noir	Purified mannoprotein	Reduced astringency	Escot et al., 2001
Tempranillo	Lees and yeast derivatives	Reduced astringency and green tannin. Improved grassy, balance, overall punctuation; especially by yeast derivatives	Del Barrio-Galán et al., 2011
Tempranillo	Lees acidified with tartaric and sulphurous acids	Lysated lees had strong effects on the mouth feel, enhancing acid and fresh sensations; acidification plus enzyme increase sweetness, fullness and mouth length perception	Fernández et al., 2011
White variety	Commercial mannoprotein	The protein content of mannoprotein was found related to flavour intensity and body balance	Ribeiro et al., 2014
Red variety	Inactivated dry yeasts preparation	Reduced astringency	Wang, 2014
Chardonnay	Yeast lees	Aging on lees improved mouthfeel and length of perceiving	
Chardonnay	Inactivated dry yeasts preparation	Increased mouthfeel and palate weight	
Verdejo	Lees and commercial yeast derivatives	Improved mouthfeel. Lowered acidity and astringency, especially by yeast derivatives	Del Barrio-Galán et al., 2011

2.6.2 Aroma and colour

The effect of aging on lees on wine aroma (Table 2.4) and colour (Table 2.5) have also been discussed in studies. Aging on lees is widely used in white wine making or making wine in new barrels. As such, it has considerable influence in determining the aromatic composition of the wine (Table 2.4). Lees has been shown to adsorb wood aromas from barrels and thus prevent other wine aromas being masked (Pérez-Coello & Díaz-Maroto, 2009). Most wood-derived compounds, such as eugenol, 4-propylguaiacol, 4-methylguaiacol, furfural, 5-methylfurfural, 5-hydroxymethlfufural,

vanillin, syringaldehyde, and oak lactones (Jiménez-Moreno and Ancín-Azpilicueta, 2007), are generated during the thermal degradation of the lignin of oak wood as a consequence of the toasting of the staves. Guaiacol and γ-nonalactone were not significantly reduced after 12 months aging on lees in oak barrel. In addition, compounds including 4-methylguaiacol, 4-propylguaiacol, eugenol, furfural, and 5-methylfurfural were reduced significantly at a concentration of 10 g/L dry lees. Other compounds required a concentration of 50 g/L dry lees to make significant reduction but this exceeded the legal dose (10-12 g/L dry lees).

Lees is also able to adsorb volatile sulphur compounds (including thiols) and volatile phenols (Table 2.4) (Palacios et al., 1997; Vasserot et al., 2003; Jiménez-Moreno & Ancín-Azpilicueta, 2007 and 2009). Palacios et al. (1997) found that 0.05 g/L of Chardonnay lees (inoculated with *Saccharomyces cerevisiae* DV10) recovered after alcoholic fermentation was able to adsorb 200 μg/L of hydrogen sulphide sufficiently, and 200 μg/L of methanethiol or ethanethiol can be adsorbed by 0.15 g/L lees.

Esters are very important for wine quality as they are responsible for fresh and fruity aromas. Esters can be produced during both alcoholic fermentation and aging (Liberatore et al., 2010). Light lees is the major source of esters during aging and the major group of volatiles released during yeast autolysis. For example, ethyl esters with fruity odours consistently increased during autolysis (Charpentier, 2010).

Aging on-lees is now a common vinification technique used in red wine to provide more rounded and less astringent mouthfeel (Rodrigues et al., 2013). This is based on a partial removal of wine polyphenols by yeast lees. However, this technique can also modify the colour of wine due to loss of wine anthocyanins (Mazauric & Salmon, 2006; Vasserot et al., 1997; Morata et al., 2003). For example, about one-third of the total content of free anthocyanins in wine was lost after one week aging on lees (Mazauric & Salmon, 2006). Investigations of the adsorption of anthocyanin to yeast cell walls have been carried out in both model solution and wine (Table 2.5). In a model wine study, Vasserot et al. (1997) attributed the interaction between yeast lees and anthocyanin to a weak and reversible adsorption of anthocyanins on yeast cell walls. In addition, anthocyanins were adsorbed in proportion to their polarity: delphinidin > cyanidin > petunidin > peonidin > malvidin (Vasserot et al., 1997). Morata et al. (2003) reported that the colour intensity of wine aged on lees reduced from 3.46 to 2.98 after contact with yeast lees, and that greater tonality was achieved with an increase in yellow tones and a reduction in blue tones.

Table 2.4. Volatile compounds adsorbed by lees and/or yeast extracts during the aging process.

Aroma compound	Description	Reference
Hydrogen sulphide	Rotten egg	Palacios et al., 1997
Methanethiol	Bad breath, flatus	
Ethanethiol (ethyl mercaptan)	Leek, onion, durian, cooked cabbage	
Thiols	Garlic	Vasserot et al., 2003
4-ethylphenol	Barnyard, medicinal, band-aid, mousy	Jiménez-Moreno & Ancín-Azpilicueta, 2009
4-ethylguaiacol	Spicy, toasted, smoky	
4-methyguaiacol	Smoky, spicy	
Furfural	Wood, almond	Jiménez-Moreno & Ancín-Azpilicueta, 2007
5-methylfurfural	Spicy, caramel	
Eugenol	Spicy, clove, oak	
Cis-oak-lactone	Oak, coconut	
Trans-oak-lactone	Oak, coconut	
Phenol	-	
m-cresol (3-methylphenol)	Sweet tarry	
Furfuryl alcohol	Caramel	

Table 2.5 Summarized table of findings in adsorption of anthocyanins by yeast cell walls.

Substrates	Compounds adsorbed	Findings	Reference
Model wine	Malvidin Peonidin Petunidin Delphinidin Cyanin	Lees possess effective decolouring ability. Two phases of adsorption of yeast lees. The interaction between anthocyanin and yeast cell walls are weak; polarity-dependent.	Vasserot et al., (1997)
Cabernet Sauvignon	Malvidin-3G Peonidin-3G Petunidin-3G Delphinidin-3G Acylated derivatives	Lees reduced colour intensity The acyl derivatives of all anthocyanins were the most strongly adsorbed, especially the p-coumayl and acetyl derivatives of peonidin and malvidin.	Morata et al., (2003)
Model Wine	Malvidin-3G Peonidin-3G Petunidin-3G Delphinidin-3G Acylated derivatives	Colour intensity is correlated to anthocyanin derivatives. It was affected by yeast strains. All strains tested showed higher percentages of anthocyanin removal with increase anthocyanin polarity	Medina et al., (2005)
Graciano	Anthocyanin & derivatives 6-p-coumaroyl 6-caffeoyl Vitisin Acetyl derivatives	The quantity of adsorbed anthocyanin was depended on yeast strains. The tonality of cell wall extracts was greater than the wine. Vitisin were weakly adsorbed.	Morata et al., (2005)
Model Wine	Glucosyl, acetyl & p-coumaroyl derivatives	The adsorption of anthocyanin was not only related to their polarity. Tannin extracted from yeast lees had high polymeric size and high percentage galloylated residues. Nonpolar tannins were preferentially adsorbed on yeast lees.	Mazauric & Salmon, (2006)

2.7 Summary

As highlighted throughout this literature review and also in the reviews by Caridi (2006) and Pérez-Serradilla and Castro (2008), there is no doubt that on-lees aging can improve the organoleptic qualities of finished wines. Polysaccharides and mannoproteins from yeast cell walls are the major chemical components responsible for the modification of wine mouthfeel, but yeast lees were also found to be able to modify aroma and colour in various ways.

Yeast autolysis is a slow progress under normal wine conditions of low pH, low temperature, and the presence of ethanol. This chapter has also reviewed different conventional and novel methods (such as stirring, the addition of enzymes, and pulsed electric field) that are used or can be used in winemaking. Stirring is a traditional method used in wine aged on lees with the requirement of significant labour input. The use of stirring in the wine industry is currently based winemakers' experiences, and the guidance is still poorly supported by scientific research. Endogenous enzymes such as pectinases and glucanases (β-1,3-1,6 glucanases) were reported to be able to speed up the yeast autolysis process (Canal-Llaubères, 2010). However, in practice low temperatures limit reaction rates. PEF is a novel technology which has been applied in the wine industry in recent years for inducing yeast autolysis, enhancing extraction and accelerating maturation. This technology has potential to improve cost efficiency and yield of winemaking. Field strength, frequency, and number of pulses are the three important operational parameters need to be considered before application.

The objectives of this study were: 1) to investigate the effects of different lees management techniques on the release of chemical compounds; 2) to determine the feasibility of pulsed electrical field for accelerating on-lees aging process; and, 3) to investigate the lexicon for the description of mouthfeel of Sauvignon blanc wine using napping®, and how these words relate to the chemical composition of wines.

Chapter 3

General Chemical and Physical Methods of Analysis

3.1 Materials

3.1.1 Wines

Twenty commercial Sauvignon blanc wines were purchased locally in October, 2015. Of these wines, 16 were from Marlborough, 2 from Hawke's Bay and 2 from the Nelson region (Table 3.1). Wines were selected in discussion with the well-known wine judge and writer, Jo Burzynska, with a view to obtaining examples representative of the diversity of styles produced in New Zealand.

For chemical analyses, 50 mL samples were taken from freshly opened bottles and transferred to GC vials (50 mL, Sigma-Aldrich, Merck). The headspace was sparged with nitrogen gas, vials were sealed and stored at 4 °C.

Table 3.1 Summary of wine used in the current study.

Brand Name	Year	Variety	Region	Series (if relevant)
Jackson Grey Ghost	2011	Sauvignon blanc	Marlborough	Barrique Sauvignon blanc
Dog point	2012	Sauvignon blanc	Marlborough	Section 94
Fairbourne	2013	Sauvignon blanc	Marlborough	
Mahana	2013	Sauvignon blanc	Nelson	
Millar Road	2013	Sauvignon blanc	Hawke's Bay	Green glow
Sound of White	2013	Sauvignon blanc	Marlborough	
Spy Valley	2013	Sauvignon blanc	Marlborough	Enovy
Spy Valley	2013	Sauvignon blanc	Marlborough	Enovy
Villa Maria	2013	Sauvignon blanc	Marlborough	Southern clay
Aronui Single	2014	Sauvignon blanc	Nelson	Single vineyard
Brancott	2014	Sauvignon blanc	Marlborough	
Brancott	2014	Sauvignon blanc	Marlborough	Terrior (Awate Valley)
Giesen	2014	Sauvignon blanc	Marlborough	
Lime Rock Coquina	2014	Sauvignon blanc	Central Hawke's Bay	
Saint Clair Block 43	2014	Sauvignon blanc	Marlborough	Pioneer block (43 degree)
StoneLeigh	2014	Sauvignon blanc	Marlborough	Latitude
Peter Yealands	2015	Sauvignon blanc	Marlborough	
Saint Clair	2015	Sauvignon blanc	Marlborough	Vicar's choice
StoneLeigh	2015	Sauvignon blanc	Marlborough	
Villa Maria	2015	Sauvignon blanc	Marlborough	Private bin

3.1.2 Wine lees

Three different lees materials were used: inactivated dry yeast, rehydrated dry yeast and collected wine lees. Opti-lees (an inactivated dry yeast) was purchased from Lallemand, Canada. Maurivin AWRI R2 was supplied by AB Mauri, Australia, and rehydrated according to the manufacturer's instruction. Yeast lees of (c. 150 kg wet weight) were collected from the Brancott Winery in Blenheim. Because there is no literature regarding storage, and lees materials starts break down under exposure to the environment once being racked off from the wine, the storage protocol was developed based on communication with a local winemaker (Andy Frost, Research Winemaker, Pernod Ricard). After collection, the wine lees was temporarily transferred to a stainless steel keg with an air-tight cap and stirred vigorously to homogenise the material. After 3 days 4 °C, the wet lees were fractionated into 4 fractions by siphoning into 4 separate plastic kegs. The wine lees in the stainless steel keg was stirred vigorously, then transferred to plastic kegs. The first 25 L plastic keg filled with the wine lees was called Fraction 1; the second and third 25 L plastic kegs were called Fraction 2 and 3, respectively. The rest of the wine lees was filled into a 50 L plastic keg (Fraction 4). The remaining solid residual in the stainless steel keg was discarded. Sulphur dioxide were added to wine lees sample to reach the minimum free SO_2 concentration of 30 mg/L. Subsequently, the lees was stirred and the free SO_2 concentration were monitored weekly and adjusted so that it was maintained at 30 mg/L throughout.

3.1.3 Chemicals

Sodium carbonate and anhydrous di-sodium hydrogen orthophosphate were from BDH (Poole, UK). Methylcellulose and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent, catechin, sodium borate, D-(+)-galactose and β-glucanase (≥1.0 units/mg solid) were obtained from Sigma-Aldrich Chemical Co. (Steinheim, Germany). Ammonium sulphate was obtained from J.T. Baker (Philipsburg, USA). Ethanol (100%), sodium hydroxide, and saturated phenol were obtained from Fisher Scientific (Loughborough, UK). Methanol (100%) was obtained from Merck (Darmstadt, Germany). Propidium iodide was purchased from Sigma-Aldrich, Auckland, New Zealand. Surphuric acid was purchased from Thermos-Fisher scientific (New Zealand). All reagents and chemicals used in this study were of analytical grade or higher.

3.2 Methods

3.2.1 Chemical analysis

Determination of alcohol

The alcohol concentration of samples was determination using an ebulliometer (Laboratories Dujardin-Salleron, Noizay, France) as described by Iland (2004). Alcohol concentration was measured in triplicate.

Determination of residual sugar

The residual of sugar concentration of wine samples was determined as described by Iland (2004). A glucose solution (10 g/L) was used as a standard to check the accuracy of the procedure.

Determination of glycerol

The glycerol concentration of wine samples was analysed using the Randox glycerol colorimetric analysis kit (GY-105) with RX-Daytona benchtop clinical chemistry analyser (Randox, Ireland). The glycerol content was determined according to the manufacturer's instruction.

Determination of polysaccharide

The polysaccharide concentration of wine samples was determined as described by Segarra et al. (1995), except that saturated phenol was used instead of 80% phenol solution. For the determination of acidic polysaccharides, the solution was incubated in a water bath for 15 minutes (at 20 °C) after addition of o-hydroxydiphenyl reagent. All measurements were performed in triplicate.

Determination of total phenolics

The micro scale protocol of the Folin-Ciocalteau colourmetric reaction as described by Waterhouse (2002) was used. A 20 µL of standard, sample or blank was added to an Eppendorf tube (2 mL) followed by addition of 1580 µL of RO water and 100 µL of Folin-Ciocalteau reagent. The solution was mixed by voltexing. A 300 µL of sodium carbonated solution was added to the mixture after an incubation of 4 minutes at the room temperature. The absorbance readings were taken at the wavelength of 765 nm on Shimadzu UV-1800 spectrophotometer after mixing and 60 minutes incubation at room temperature. The concentration of total phenolic was calculated from a standard curve constructed using gallic acid solutions (standard concentration was between 0 mg/L to 1000 mg/L).

Determination of total tannin

The total tannin content of extracts of freeze-dried wine lees were determined using the

methylcellulose precipitable (MCP) tannin assay as described by Sarneckis et al (2006). This is the Australian Wine Research Institute (AWRI) standard method for measuring the total tannin content of grape homogenates and red wines. The assay total volume was 1 mL for both treatment and control test. For sample measurement, 100 µL of extract was transferred to Eppendorf tube followed by 300 µL of 0.04% methylcellulose (MC), 200 µL of saturated ammonium sulphate (SAS) and 400 µL of de-ionised water (DI). The tubes were left at room temperature for 10 minutes, followed by centrifugation at 2890 g for 5 minutes. The absorbance of the resultant supernatant was read using an UV spectrophotometer at the absorbance of 280 nm. For the control, the addition of MC was replaced by adding DI. Epicatechin was used as a standard, and the range of epicatechin standard concentration was 0, 25, 50 75 and 100 µg/mL. For calculation of tannin content, the absorbance difference between control and treatment was substituted in a linear equation generated from epicatechin standard curve. The obtained result was then divided by 10 (dilution factor). The tannin content was then reported on epicatechin equivalent basis (mg epicatechin eq/g FD lees).

Determination of total protein

The Bradford method was used for the determination of the total protein concentration of wines samples as described by instruction manual of the Bio-Rad dye reagent (Bio-Rad, New Zealand). Bovine serum albumin (BSA) solution with the range between 0 to 50 mg/L was used to construct the calibration curve. The analysis was carried out on a microplate. Each standard or sample solution of 10 µL was added into separate microplate wells followed by the addition of 200 µL of diluted dye reagent (1 part dye reagent concentrate with 4 parts RO water). Sample and reagent were mixed thoroughly using a microplate reader mixer. The absorbance was measured at 595 nm using FLUstar Omega microplate reader (BMG Labtech, Germany). All measurements were performed in triplicate.

Determination of primary amino acid nitrogen

Primary amino nitrogen (PAN) in wine samples were determined using the PAN analysis kit (Megazyme, Ireland). The PAN content of wine sample was determined according to the manufacturer's instructions. The absorbance reading was taken at the wavelength of 335 nm by a Shimadzu UV-1800 spectrophotometer. Sample and reagent were mixed thoroughly using a microplate reader mixer. The absorbance was measured at 595 nm using FLUstar Omega microplate reader (BMG Labtech, Germany). All measurements were performed in triplicate.

Determination of free and total amino acids

Free amino acids were determined as described by Guilloux-Benatier and Chassagne (2003). Several modifications were made. The total and free amino acids were analysed by using Agilent 1100 Series HPLC system equipped with a 250 mm × 4.6 mm, 5 µm Prodigy column (temperature: 40°C)

(Phenomenex) and a fluorescence detector. The excitation and emission of the fluorescence detector were set at 335 nm and 440 nm, respectively. For the detection of proline and hydroxyproline, the detector was switched to another channel at 26 mins (excitation: 260 nm; emission: 315 nm). O-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) were used as the derivatization reagent for primary and secondary amino acids, respectively. The volume (11 µL) of reagents was injected by using an auto-sampler. Two mobile phases (A and B) were used. Mobile phase A was 0.01 M Na_2HPO_4 solution (pH = 7.5) with 0.8% of tetrahydrofuran (THF). Mobile phase B consisted of 20% Mobile phase A (v/v), 40% of methanol (v/v) and 40% acetonitrile (v/v). The gradient of mobile phase A and B is shown in Table 3.2.

Table 3.2 Pump Gradient of mobile phase A and B for determination of free and total amino acids.

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	1.0	100	0
14	1.0	60	40
22	1.0	45	55
27	1.0	0	100
35	1.0	0	100
36	1.0	100	0
40	1.0	100	0

Total amino acids were determined in the same way as free amino acids after hydrolysis. The acid hydrolysis was performed as described by Alexandre et al (2001).

Determination of mannoprotein

The concentration of mannoprotein in wine samples was measured as described by Quirós et al (2012) with modifications. Samples (2 mL) were filtered by using Zeba spin desalting columns (Thermo Scientific). The filtration was done twice such that each sample was passed through two separate desalting columns. After hydrolysis, the reconstituted samples were filtered through 0.22 µm pore sized nylon filters. The filtered samples were then analysed by using a Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with an ELSD detector (model-3300, Alltech). PrevailTM carbohydrate ES column (250 mm × 4.6 mm) with guard column was used for separation. Mobile phase A (acetonitrile) and B (RO water) were used; the concentration and flowrate of mobile phases are showed in Table 3.3. The ELSD detector was set at flowrate: 1.4 L/min and temperature of 38°C.

Table 3.3 Pump gradient of mobile phase A and B for determination of mannoprotein.

Time (min)	Flow rate (ml/min)	Column temperature (ºC)	Mobile phase B (%)
0-5	1.0	20	20
5-13	1.0	20	20-50
13-14	1.0	20	50-20
14-19	1.0	20	20

Using this method, mannose was successfully separated from the other sugar monomers that can be found in wine such as fructose, glucose, galactose and trehalose. However, galactose was not able to be separated from glucose due to its similar chemical structure and molar mass (Figure 3.1). Therefore, galactose was quantified as glucose in the current study. The content of mannose and glucose were used to represent the concentration of mannoprotein and β-glucan according to the method described by Quirós et al. (2012).

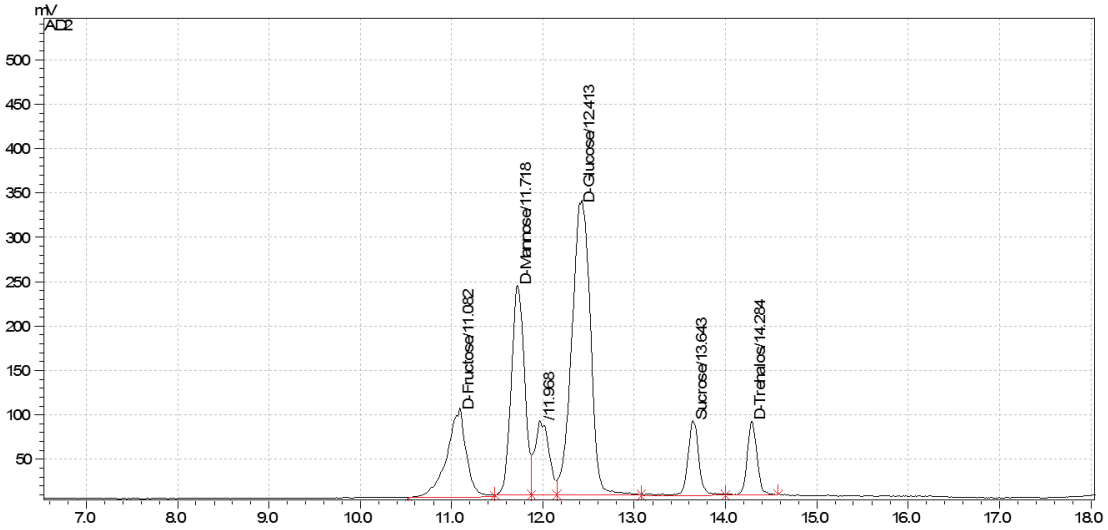


Figure 3.1 Example of the chromatogram of a sugar mix.

3.2.2 Physical analysis

Monitoring cell lysis

Cell lysis by PEF was monitored as described by Martínez et al. (2016). Untreated and PEF treated samples were measured at different absorbances using a Shimadzu UV-1800 spectrophotometer. The turbidity of the yeast suspension were measured at 600 nm for monitoring the leakage of cellular content. The supernatants were measured at 260 nm and 280 nm for monitoring the

presence of nucleic acids and proteins released from yeast cells, respectively.

Determination of the percentage of permeablilized cells

The percentage of permeabilizaed cells were determined as described Martínez et al. (2016) with modifications. Propidium iodide (PI) is a fluorescent dye which was used for straining permeabilized cells. For staining yeast cells, 50 µL of PI (0.1 mg/mL) was added to 450 µL of *S. cerevisiae* suspension before and after PEF treatment, followed by an incubation at room temperature for 10 minutes. The excess amount of PI was removed by discarding the supernatant after a centrifugation at 1000 g for 5 minutes. The yeasts was then re-suspended into 500 µL of de-ionized water for further washing steps. The yeast suspension was centrifuged, and the supernatant was discarded. This washing step was repeated three times. The washed yeasts were re-suspended in 500 µL of de-ionized water, and fluorescence was measured with a spectrofluorophotometer (FLUOstar Omega microplate reader, BMG Lab Tech, Ortenberg, Germany) at 535 nm and 625 nm for excitation and emission, respectively. The percentage of permeabilized cells was based on the fluorescence intensity of for cells permeabilized by the most intense PEF treatment (10 KV/cm for 30 s).

Determination of specific gravity

The specific gravity of wine was measured as described by Pomeranz and Meloan (2000). A 10 mL specific gravity bottle (H. J. Elliott Ltd, UK) was used, and all wine samples were measured at room temperature (20°C).

Determination of viscosity

The viscosity of wine samples was determined as described by Neto et al (2014). The rheological tests were carried out in a stress-controlled rotational rheometer, model MCR 301 (Anton Paar, USA), using a coaxial double gap geometry (DG26.7) (gap width: 0.42 mm and 0.46 mm; sample volume: 3.8 mL). The rheometer was set up to using the Newtonian model; measurements were carried out following an increasing sear rate ramp in the range of 1-100 s^{-1} at the temperature of 20°C. All measurements were carried out in triplicate.

Chapter 4

Effect of Different Lysis Treatments on Chemical Components Released by Three Different Yeast Treatments during Aging on-Lees in a Model Wine

4.1 Introduction

Aging on lees is a traditional winemaking practice originally applied to selected white wines (*grands crus*) from Burgundy as well as some styles of sparkling wine. It is often used to produce a wine with an enhanced structure and mouthfeel, extra body and increased aromatic complexity (Charpentier, 2010). During on-lees aging, yeast components are released as a consequence of enzymatic cell self-destruction called yeast autolysis. Among those components released, mannoproteins, amino acids, proteins, and nucleotides are responsible for wine mouthfeel, aroma, sweet and bitter tastes, and flavour, respectively (Alexandre & Guilloux-Benatier, 2006). Autolysis is highly dependent on the ambient temperature and pH with ideal conditions reported to be pH 5 at 45°C (Charpentier & Feuillat, 1993). In wine, autolysis occurs in less than ideal conditions of with pH values around 3 and temperatures of approximately 13°C. Different vinification techniques have been used during on-lees aging, such as periodic stirring and the addition of β-glucanase to reduce the time required; more recently, the addition of inactivated dry yeasts has also been used for this purpose. In the wine industry, the use of these methods is based on the experience of winemakers as there is limited scientific information for guidance. Nevertheless, various studies have been conducted to investigate the release of yeast components to white, red and model wines (Rodrigues et al., 2012; Wang, 2014; Guilloux-Benatier & Chassagne, 2003; Del Barrio-Galán et al., 2011), although the effects of periodic stirring, addition of β-glucanase, and addition of inactivated dry yeast and their interactions on release of yeast components remain poorly investigated.

The objective of this study was 1) to investigate the release of chemical compounds from different lees material, and 2) to investigate the effect of conventional aging methods (enzyme addition and periodic stirring) during aging in the model wine.

4.2 Experimental design

A factorial experiment incorporated three different lees materials (R, rehydrated yeasts; I, inactivated yeasts; L, collected lees), two enzyme dosages (0 and 7.5U of β-glucanase) and three stirring frequencies (every 2, 4 and 8 days) was set up. The experiment was carried out in triplicate

using a model wine. Model wines were incubated in the Contherm Precision Environmental Chamber (54000RHS, Contherrm Scientific Ltd, New Zealand) at 20 °C for 160 days. Samples were divided into three blocks, and the location of each sample within the incubator was randomized to avoid the effects of cold or hot spots.

4.3 Materials and methods

4.3.1 Preparation of model wine

A model wine solution was prepared as described by Martínez-Rodríguez and Polo (2000). The model wine solution contained ethanol (10%, v/v), tartaric acid (4 g/L), malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate (0.025 g/L). The pH was adjusted to 3.0 with sodium hydroxide. Model wine treatments were pre-poured in 1 L Schott bottles. The total number of bottles was 54. Every bottle was given a unique bottle code.

4.3.2 Addition of wine lees

Details of the wine lees materials and their preparation are given in Chapter 3 (Section 3.1). Collected lees were pre-washed before addition to the model wine. The collected lees were firstly centrifuged at 4500 g for 15 mins and the supernatant was discarded. The pellet was washed three times with 0.9% (w/v) NaCl solution as described by Guilloux-Benatier and Chassagne, (2003). Model wine solution was used to re-suspend the washed collected lees before addition to the model wine. The other wine lees materials were added directly to the model wine after preparation. All lees materials were added to model wine for a final concentration of 5% (w/v).

4.3.3 Enzyme addition, stirring and sampling

Two enzyme dosage rates were utilized, 0 U and 7.5 U. The enzyme solution was prepared by dissolving 300 mg of β-glucanase (Sigma-Aldrich, USA) in 2 mL of model wine solution. For the enzyme dosage of 7.5 U, 50 µL of the enzyme solution was added to model wines by pipetting. For the enzyme dosage of 0 U, an addition of 50 µL of model wine solution was used instead of the enzyme solution.

Model wines were stirred using an orbital shaker (MaxQ 4000, Thermo Scientific, USA) for 10 minutes at 150 rpm in an incubator. Samples (20 mL) were taken after stirring on every 8th day. After stirring, 20 mL of sample was transferred to a 50 mL centrifuge tube by pipetting. After sampling, the model wine was immediately sparged with nitrogen (flowrate: 2L/min) for 15 seconds. Samples were then centrifuged at 4500 g (Dupin et al., 2000), and subsequently the supernatant was

subdivided into centrifuge or Eppendorf tubes, and stored at -20 °C.

4.3.4 Chemical and physical analysis

Materials are described in Chapter 3 (Section 3.1). The concentrations of total protein, primary amino acid nitrogen, free amino acids, neutral and acidic polysaccharides, mannoprotein, glucan, and glycerol, as well as analysis of viscosity were determined as described in Chapter 3 (Section 3.2).

4.3.5 Statistical analysis

Analysis of variance (ANOVA) of the repeated measurements was calculated using Genstat 16 (VSN International Ltd, UK). Statistical results for total protein concentration are used as an example to illustrate the output from this analysis (Appendix I). Principle Component Analysis (PCA) was performed using XLSTAT (2017, Microsoft, USA).

4.4 Results and discussion

4.4.1 Total protein concentration

The total protein concentration was determined for an aging period of 56 days. Samples were not collected after day 56 because values appeared to have plateaued. Overall, lees material, stirring frequency, enzyme treatment, time and most interactions had a significant effect ($P < 0.05$) on total protein concentration in the model wine (Table 4.1).

To illustrate these results, Figure 4.1 shows the change in the total protein concentration of model wine containing different lees material stirred every 4 days throughout the aging experiment. The total protein concentration on day 1 were different in R, L and I treatments. Subsequently, an increase in protein concentration was observed in the R treatment with no added enzyme during the first eight days; then the protein concentration decreased and reached a plateau on day 48. In comparison, less variation in total protein concentration was observed in all the other treatments. The time course of total protein concentration in both L treatments and I treatments followed a similar pattern with lower protein concentrations in for the L compared to R treatment throughout the whole period. The low protein concentration for L was expected due to the original condition of the collected lees which had been stored and pre-washed with 0.9% NaCl (v/v) before adding to the model wine; this would have removed nitrogenous compounds (protein) from the lees.

Table 4.1 Results of ANOVA for total protein concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Enzyme	< 0.001
Lees material	< 0.001
Stirring frequency	0.013
Enzyme.Lees material	< 0.001
Enzyme.Stirring frequency	< 0.001
Lees material.Stirring frequency	< 0.001
Enzyme.Lees material.Stirring frequency	0.002
Block.Subject.Time stratum	
Time	< 0.001
Time.Enzyme	< 0.001
Time.Lees material	< 0.001
Time.Stirring frequency	< 0.001
Time.Enzyme.Lees material	< 0.001
Time.Enzyme.Stirring frequency	0.006
Time Lees material.Stirring frequency	0.001
Time.Enzyme.Lees material.Stirring frequency	0.151

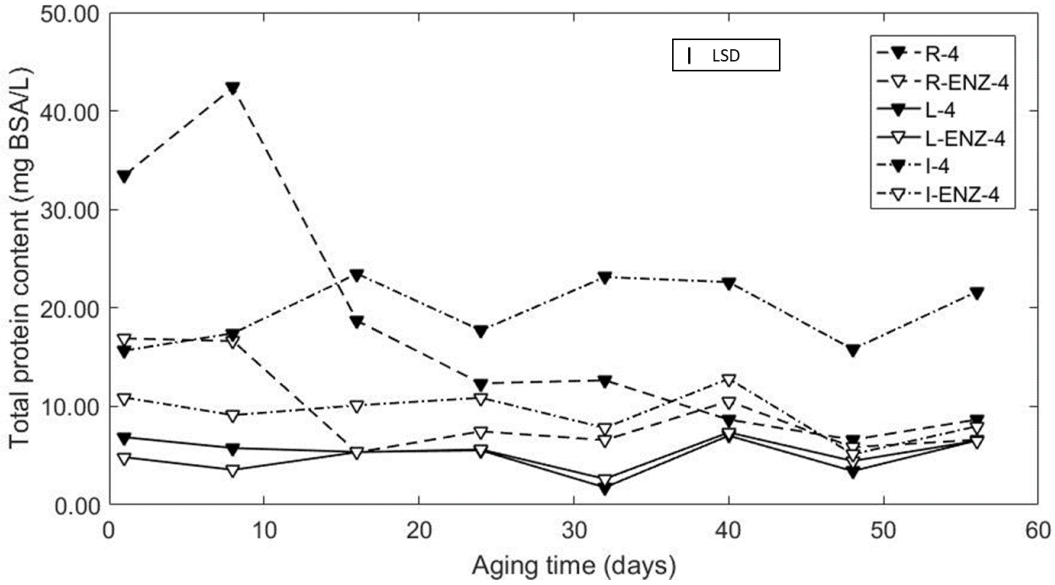


Figure 4.1 Total protein concentration of model wines containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 9).

The addition of enzyme significantly affected the release of proteins from different lees material throughout the aging experiment ($P < 0.05$). There was a consistent trend for total protein concentration to be greater in the absence of additional enzyme. Although stirring frequency, and its interactions with time showed a statistical significant impact on the total protein concentration in model wine, the magnitude of this effect was small compared with the effect of other treatments (data not shown).

Nunez et al (2005) studied autolysis of yeast (strain IFI473 and its mutants) in sparkling wine and found protein and polypeptide changes during wine aging were significantly correlated with the time of aging; for example, the protein concentration increased during the first one or two months of aging, followed by a decrease. This is consistent with the finding in the current study.

Few studies had been carried out using propagated or rehydrated yeasts. In the current study, the large increase in protein concentration in the model wine containing rehydrated yeasts at the beginning of aging experiment might be attributed to a combination of a passive exorption (the phenomenon of excretion) and a release of mannoprotein. Both Zhao and Fleet (2003), and Fornairon-Bonnefond and Salmon (2003) carried out short term experiments (10 and 20 days, respectively) in either buffer or synthetic wine. Propagated yeast cells were used in both studies. Zhao and Fleet (2003) used lees that had no yeast cell viability, and the lees used by Fornairon-Bonnefond and Salmon (2003) was harvested at the end of exponential growth with a final

concentration of 108 – 109 cells/mL in synthetic wine. The protein concentration in both studies was found to increase rapidly in the first day of the autolysis experiment. In other studies, monitoring of yeast autolysis during on-lees aging were done by determining total nitrogen using the Kjeldahl method (Leroy et al., 1990; Martínez-Rodríguez & Polo, 2000). In a 15-day autolysis experiment with rehydrated yeasts (10 g/L), total nitrogen concentration increased from less than 10 mg/L to 234 mg/L within 20 hours. Observations in previous studies suggest that exorption of intracellular protein occurred almost immediately once yeast cells are introduced to model wine. In the current study, the change in total protein concentration was not monitored during the first 24 hours. The first readings of total protein concentration in all treatments were not zero, which can be attributed to the exsorption mechanism. The further increase in total protein concentration might be attributed to the release of mannoprotein after exsorption of the rehydrated yeast cells. A decrease of total protein concentration after day 8 can be attributed to the enzymatic breakdown of proteins. Martínez-Rodriguez et al. (2001) reported a similar trend and suggested that proteins break down to fragments with molecular weight less than 3500 Da that do not react with Coomassive Blue dye and hence are not detected by the Bradford method for determination of protein.

4.4.2 Amino acids

4.4.2.1 Total free amino acids

The total free amino acid concentration of the model wine containing different lees material was determined for an aging period of 56 days, the same as for total protein concentration. Lees material, but not enzyme addition and stirring frequency, had a significant effect ($P < 0.05$), although there were significant one-way interactions between enzyme addition and stirring frequency and between lees material and stirring frequency (Table 4.2). Time was also a significant factor, but significant interactions of time with all other factors and interactions suggested a very complex evolution in the total free amino acid concentrations. Even though these interactions with time showed a statistical significant impact on total free amino acids in model wine, the magnitude of these effects were small compared with the other treatments.

Table 4.2 Results of ANOVA of total free amino acids and primary amino acid N in model wines with different treatments.

Source of variation	F probability	
	Total free amino acids	Primary amino acid N
Block.Subject stratum		
Enzyme	0.312	< 0.001
Lees material	< 0.001	< 0.001
Stirring frequency	0.178	< 0.001
Enzyme.Lees material	0.013	< 0.001
Enzyme.Stirring frequency	< 0.001	< 0.001
Lees material.Stirring frequency	< 0.001	< 0.001
Enzyme.Lees material.Stirring frequency	0.084	0.084
Block.Subject.Time stratum		
Time	< 0.001	< 0.001
Time.Enzyme	0.004	< 0.001
Time.Lees material	< 0.001	< 0.001
Time.Stirring frequency	< 0.001	< 0.001
Time.Enzyme.Lees material	0.004	< 0.001
Time. Enzyme.Stirring frequency	0.017	< 0.001
Time Lees material.Stirring frequency	< 0.001	< 0.001
Time.Enzyme.Lees material.Stirring frequency	0.028	0.079

As with total protein, concentrations of free amino acids were not monitored during the first 24 hours, and the non-zero results for the R and I treatments can be attributed to the exsorption mechanism previously described (Figure 4.2). In addition for I, damage to cell walls during the inactivation process likely allowed a rapid release of protein, polypeptides, peptides and amino acids (Pozo-Bayón et al., 2009). Also, the strain used for manufacturing the inactivated yeasts might contain more soluble free amino acid than rehydrated yeasts (EC1118). Perrot et al. (2002) studied the release of amino acids in a model wine aged on rehydrated *Saccharomyces cerevisiae* yeasts and reported that, after 8 days of aging, model wine containing strains MC001had a higher concentration of free amino acid (417 µmol/g initial dry matter) than MC002 (372 µmol/g initial dry matter).

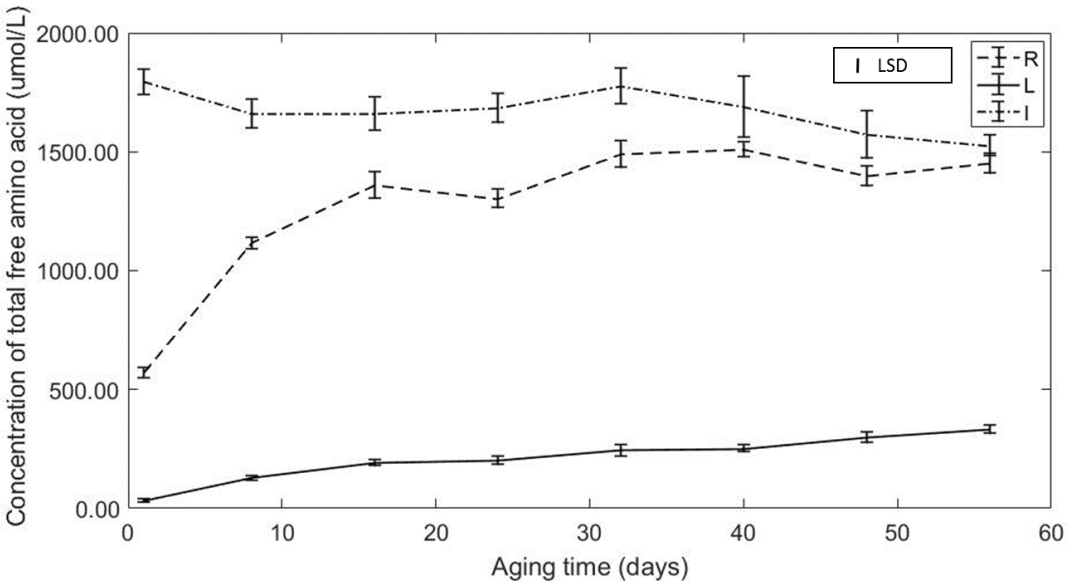


Figure 4.2 Total free amino acids concentration in model wine containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 18)

After day 1 there was a slight decreasing trend in total free amino acid concentration for treatment I (approximately 1700 µmol/L at day 1 to 1500 µmol/L at day 56), and increasing trends R (from 600 to 1500 µmol/L) and L (from 0 to 250 µmol/L). Amino acids are considered to be a good markers for yeast autolysis (Torresi et al., 2014). Martínez-Rodríguez and Polo (2000), Valles et al. (2005) and Torresi et al. (2014) reported the effect of aging time on total free amino acid concentration for *Saccharomyces cerevisiae* yeasts into model wine, sparkling wine and cider, respectively. For example, Valles et al. (2005) found that total free amino acids increased from 18 mg/L at day 22 to 40 mg/L after 11 months, and was then stable until the end of the aging experiment at 26 months. Various studies have demonstrated proteolytic activity during yeast autolysis using total free amino acids concentrations (Torresi et al., 2014; Bozdoğan & Canbaş, 2012; Valles et al., 2005; Martínez-Rodríguez & Polo, 2001).

On day 56, the concentration of free amino acids in the model wine containing R (1500 µmol/L) was higher than that containing L (250 µmol/L). This was contrary to the finding reported by Perrot et al. (2002), in which after aging of 8 days a model wine containing the rehydrated yeasts had a lower concentration (417 µmol/g initial dry matter) than one containing yeasts grown in wine (similar to the collected lees in the current study) (1964 µmol/g initial dry matter). Most likely this can be attributed to the loss and removal of amino acids in the pre-washing step as the total amino acids concentration in the supernatant from this pre-wash step was 75 mmol/L.

The decreasing trend in the concentration of total free amino acid in the model wine containing I was consistent with the mechanisms of deamination reactions of amino acids or participation in the formation of different compounds described by Alexandre and Guilloux Benatier, (2006). In contrast to the decrease trend for I, the increase in total free amino acid concentration was observed for R and L. The increasing trend of the concentration of total free amino acid in the model wine containing R was likely due to passive exsorption and the hydrolysis of nitrogenous compounds (Martínez-Rodríguez & Polo, 2000; Guilloux-Benatier & Chassagne, 2003). The increasing trend for L can be attributed to the hydrolysis of nitrogenous compounds only because the exsorption mechanisim is only relevant to live cells.

4.4.2.2 Primary amino acid nitrogen (PAN)

Separately from total free amino acids, primary amino nitrogen (PAN) concentrations were also determined. Similar to the results for total free amino acids, lees material, stirring frequency, and enzyme treatments, time and most interactions had a significant effect ($P < 0.05$) (Table 4.2). Although stirring frequency, and its interactions with time and/or enzyme addition showed a statistical significant impact on PAN, the magnitude of these effects were small compared with the other treatments.

The greatest PAN concentrations were observed in the R treatment (up to 60 mg/L); concentrations determined in treatment I were much lower (approximate 35 mg/L), and the L treatment had the lowest concentrations among three lees material. The low PAN concentrations in treatment I can be attributed to loss of integrity of yeast cells, e.g. nitrogen compounds of inactivated yeasts were lost during the inactivation process. Similarly nitrogen compounds were likely lost during the washing step in the preparation of treatment L.

Figure 4.3 illustrates the correlation between the concentrations of primary amino nitrogen and total free amino acids for the different lees material treatments. As with total free amino acids, lees material is the major factor to affect PAN. There was a strong correlation of PAN with total free amino acids in both treatment R and treatment L, with an R-square value of approximately 0.80 ($P < 0.05$). Thus, PAN increased along with the release of free amino acids from these lees materials. In treatment I, PAN was poorly correlated to total free amino acids with an R-square value of 0.23 ($P > 0.05$). This indicated that PAN remained stable along with the release of free amino acids from the inactivated yeasts. This can be attributed to the loss of cell integrity of I, which is designed to give a fast release of intracellular material.

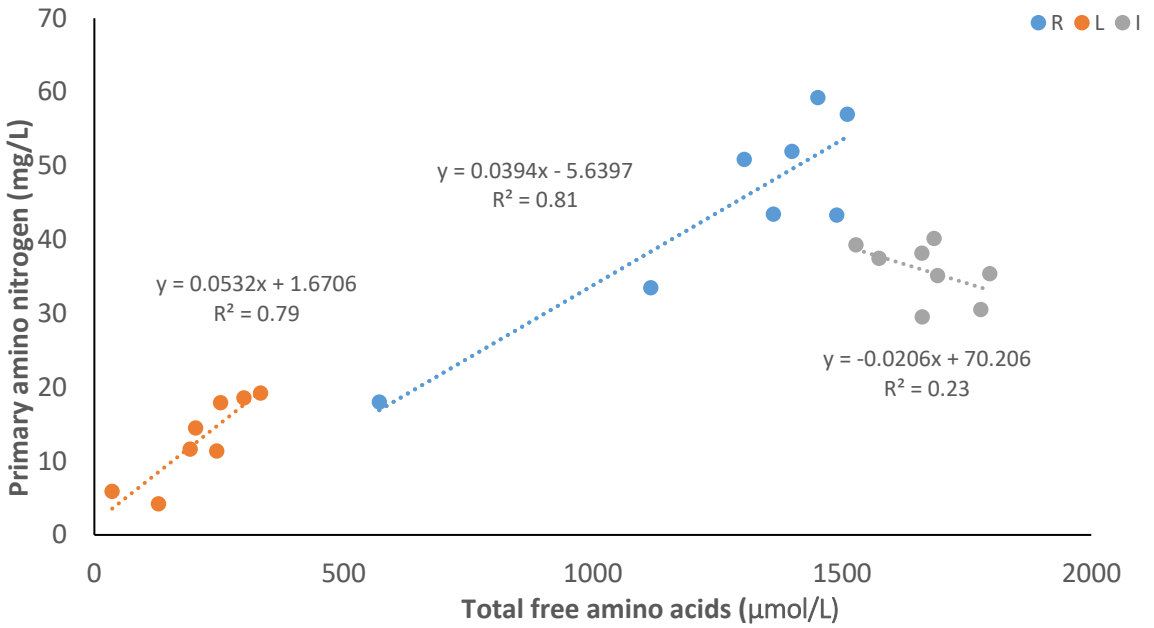


Figure 4.3. Regression analyses of the concentrations of primary amino nitrogen and total free amino acids in model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts (I) during aging experiment.

4.4.2.3 Comparison of free amino acids profile

Principal components analysis (PCA) was used to investigate the variability of the free amino acid composition of the model wines with the different lees treatments (Figure 4.4). The first two principal components explained 57% and 32% of the variance in the dataset, respectively.

According to Figure 4.4, the variation in the free amino acids composition of different lees materials can be easily distinguished. In general, samples containing rehydrated (R) and inactivated yeasts (I) had positive values on F1. In addition, all samples containing I had positive values on F2. In contrast, all samples containing collected lees (L) had a negative values on F1 and F2. These results indicate the samples from each material (R, I, L) were different in terms of the concentrations of amino acid released, which can likely be attributed to the differences between different yeast strains.

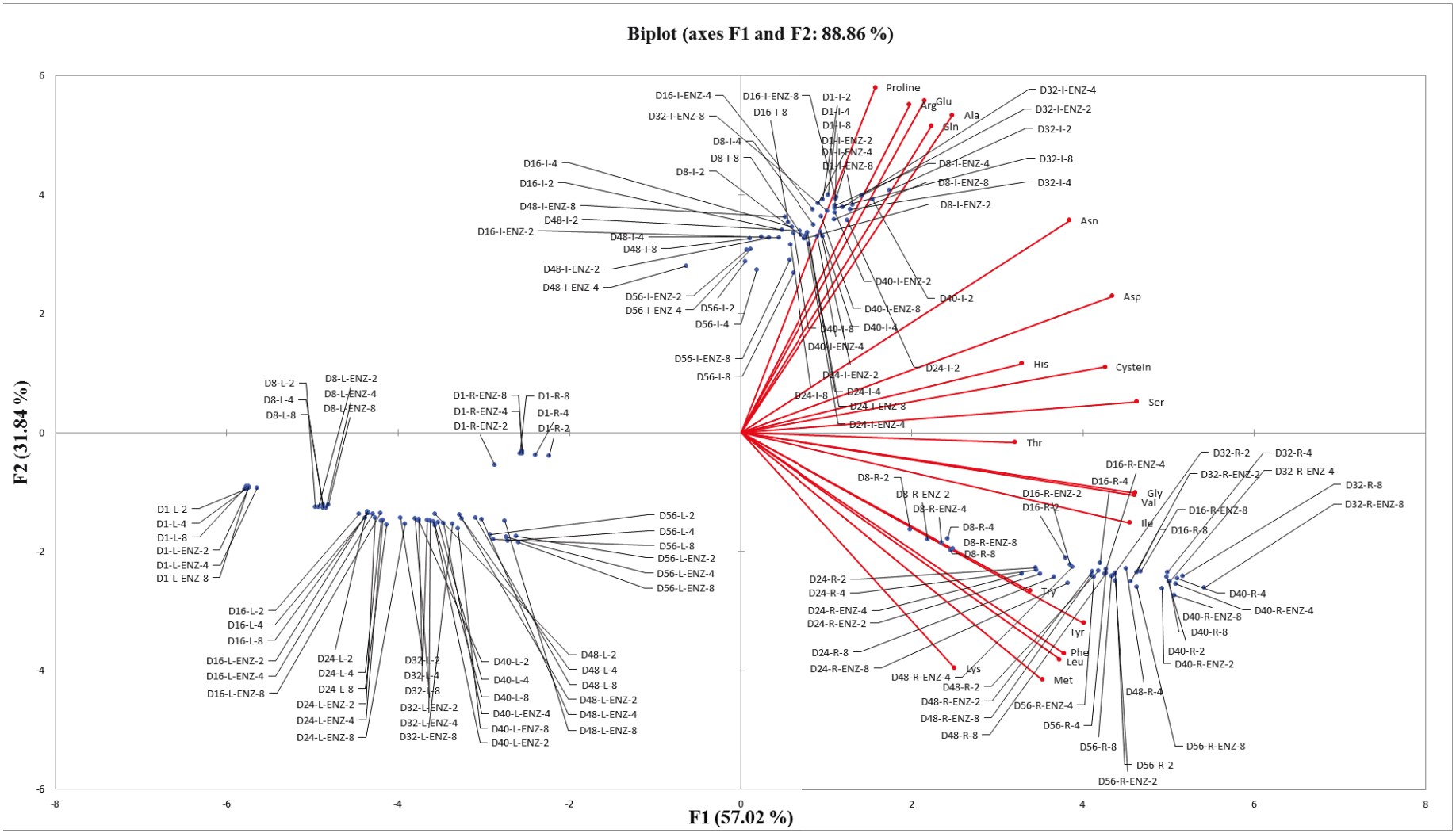


Figure 4.4 Principal component analysis (PCA) of the effect of lees treatments on the free amino acids profile of the model wines.

In more detail, samples obtained from treatment I contained higher concentrations of Ala, Arg, Gln, Glu and Pro, whereas those from treatment R contained higher concentration of Gly, Ile, Leu, Lys, Met, Phe, Try, Tyr, Thr, and Val.

In addition, Figure 4.4 illustrates the variation of the free amino acid composition at different times during aging. For R and L, samples from day 1 and 8 were well separated from the samples from day 16 to 56. The concentration of the different free amino acids generally increased with time, and the composition of free amino acids changed. Thus, Ile, Phe, Thr, Try, and Tyr became the major amino acids toward to the end of the aging experiment. For I, samples collected from different aging times were poorly separated, indicating that, on average, variation in terms of different amino acids was limited throughout the aging experiment.

The PCA results also illustrated that the stirring and enzyme addition treatments seem to have no effect on free amino acid composition throughout the aging experiment, because poor separations were observed between the samples with stirring and enzyme addition.

4.4.2.4 Change of free amino acids concentration during yeast autolysis

Time and its two-way interactions with lees material and stirring frequency had significant impacts on all amino acids ($P < 0.05$) (Table 4.3). Interaction between time, enzyme and stirring frequency were not statistically significant except for Arg, Gly, His, Ile, Lys, Met and Phe. In addition, the interaction between all treatments was found not influence amino acids significantly except His, Lys and Met.

Table 4.3 Results of ANOVA for individual amino acids.

Amino acid	Time	Time Enzyme Stirring frequency	Time Lees material Stirring frequency	Time Enzyme Lees material Stirring frequency
Ala	<0.001	0.219	<0.001	0.182
Arg	<0.001	0.002	<0.001	0.164
Asn	<0.001	0.195	0.008	0.294
Asp	<0.001	0.67	<0.001	0.948
Cys	<0.001	0.083	<0.001	0.075
Gln	<0.001	0.184	<0.001	0.37
Glu	<0.001	0.123	<0.001	0.109
Gly	<0.001	0.016	<0.001	0.537
His	<0.001	<0.001	<0.001	<0.001
Ile	<0.001	0.046	<0.001	0.283
Leu	<0.001	0.1	<0.001	0.66
Lys	<0.001	0.011	<0.001	<0.001
Met	<0.001	0.031	<0.001	0.408
Phe	<0.001	0.016	<0.001	0.033
Pro	<0.001	0.517	<0.001	0.266
Ser	<0.001	0.116	0.002	0.226
Thr	<0.001	0.308	<0.001	0.154
Try	<0.001	0.652	<0.001	0.651
Tyr	<0.001	0.109	<0.001	0.51
Val	<0.001	0.182	<.0001	0.611

Table 4.4 reports the molar percentage of each amino acid at the beginning and end of aging for each lees material. The molar distribution of the free amino acids was used to identify predominant amino acids (Moreno-Arribas et al., 1998), and also facilitated the comparison between the different samples.

Table 4.4 Amino acids distribution (expressed as a molar percentage) in the model wine containing different lees material at Day 1 and Day 56 in aging.

Amino acids	R		L		I	
	Day 1	Day 56	Day 1	Day 56	Day 1	Day 56
Ala	14.76	11.12	6.55	6.30	25.74	22.63
Glu	27.12	11.20	8.19	5.84	28.82	34.82
Leu	3.66	11.48	9.76	13.84	1.59	1.63
Ser	5.41	4.11	2.60	2.38	2.27	2.47
Thr	2.59	11.51	3.13	2.57	3.83	0.96
Pro	2.65	2.71	23.72	3.29	7.02	7.03
Asp	3.11	4.47	4.09	4.19	3.90	3.47
Cys	9.87	3.65	2.74	0.00	3.17	3.09
Asn	5.52	5.04	4.24	5.40	6.01	5.18
Gln	2.56	1.37	1.66	1.39	2.56	4.72
His	0.65	0.42	0.28	2.27	0.30	0.39
Gly	2.61	3.32	3.88	2.76	1.66	1.34
Arg	3.08	2.28	6.98	9.94	5.13	4.20
Tyr	1.62	3.09	2.59	3.94	0.78	0.65
Val	5.48	6.67	4.14	3.99	2.53	3.02
Met	0.67	1.87	1.73	2.67	0.16	0.23
Trp	0.26	0.29	1.01	0.55	0.22	0.18
Phe	2.24	7.13	5.84	8.51	1.03	1.09
Ile	2.86	4.99	3.46	3.43	2.03	1.84
Lys	3.26	3.28	3.40	16.74	1.26	1.06

R: Rehydrated yeasts; L: Collected lees; I: Inactivated yeasts.

On this basis, on day 1, Ala and Glu were the most prevalent free amino acids in the model wines containing R and I, and combined constituted 42% and 55%, respectively of the total. In addition, Cys (10%) and Ser (5%) were also important for treatment R, and Pro (7%), Asn (5%) and Arg (5%) were important for treatment I. For the L treatment, Pro and Leu were the most prevalent free amino acids constituting 24% and 10% of the total, respectively, followed by Glu (8%) and Ala (7%).

As already stated above, the distribution of free amino acids for I was similar on day 1 and day 56, but major changes in the distribution of free amino acids were found for R and L. Thus, Ala + Glu decreased from 42% to 22%, and Cys decreased from 10% to 4%, while Leu increased from about 4% to 11%. This is consistent with the results reported by Perrot et al. (2002) in that Leu became the one of most prevalent amino acids after 188 hours of autolysis. At day 56, the concentration of Ala was significantly higher for R (150 µmol/L) compared L (30 µmol/L). Opposite results were found for Leu, with the highest concentration was found in the sample containing R (170 µmol/L), followed by L (50 µmol/L).

Cys was a significant proportion only in model wines containing R. It was a minor amino acid in I compared with R, and the lowest concentration of Cys was found in L, with a concentration < 5 µmol/L throughout the aging experiment. At day 1, the concentration of Cys in the samples containing R and I were similar (about 55 µmol/L). During aging, the change of Cys in R and I were very similar. At day 56, there were no significant difference between the concentration of Cys in R and I (about 60 µmol/L). The identification and quantification of Cys were commonly not reported in previous studies in model wine and sparkling, which might be due to its low concentration towards to the end of aging.

Glutamic acid (Glu) had been found related to umami tastes in wine which also give a sense of fullness and roundedness in the mouth (Klosse, 2012). Glu was prevalent in model wines containing R and I, remaining fairly stable in concentration throughout aging experiment. At day 56, the concentration of Glu in I (480 µmol/L) was significantly higher than R (150 µmol/L).

Based on these results, the distribution of free amino acids during yeast autolysis seems to be influenced predominantly by aging time and yeast strain. The prevalent amino acids varied in concentrations were Ala, Cys, and Leu. In another study of the release of free amino acids from rehydrated yeasts (*S. cerevisiae* MC001 and MC002 strains), Perrot et al. (2002) found that Glu, Ala, Asp and GABA were the most prevalent free amino acids. Differences might be attributed to the use of the different yeast strains and autolytic conditions (pH of model wine, 3.5; stirred at 200 rpm for 8 days).

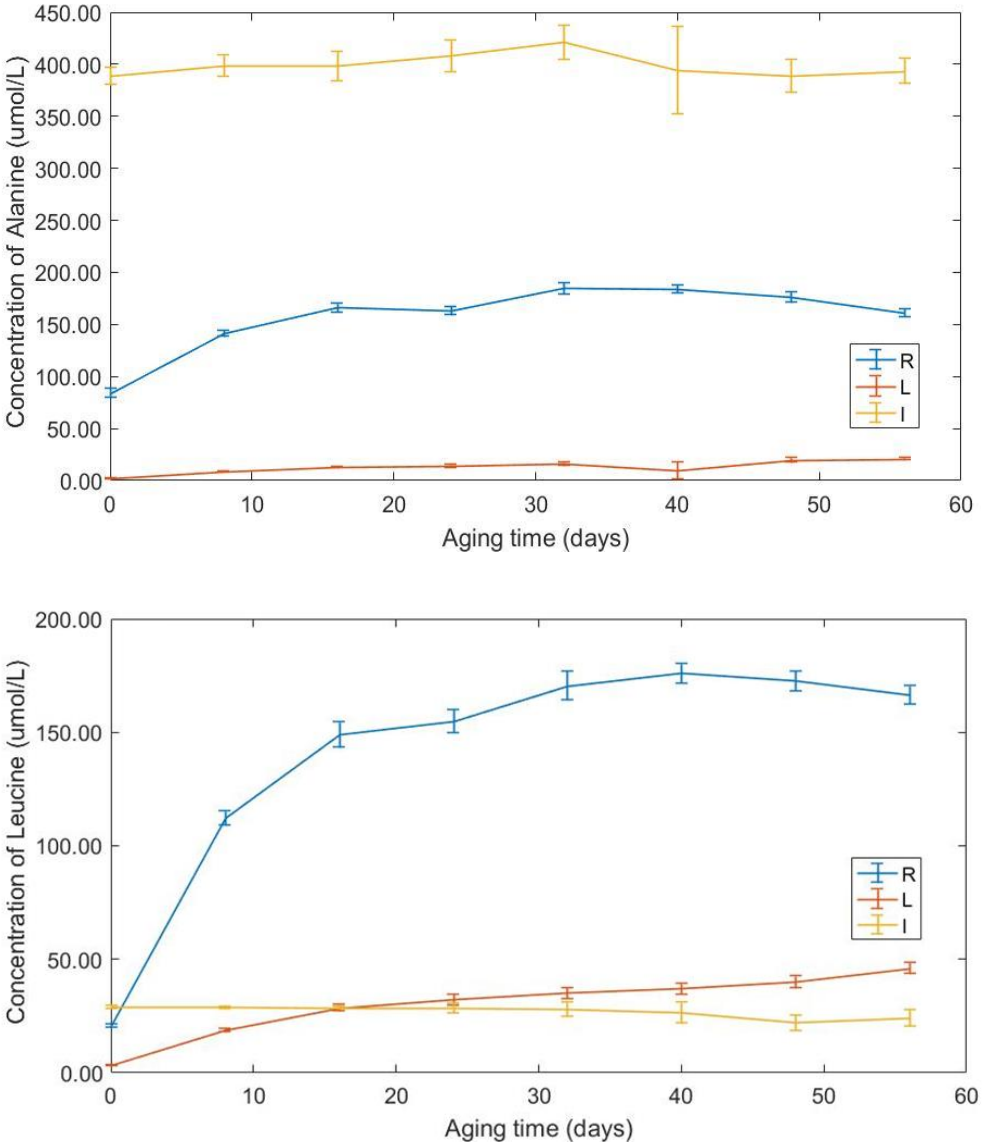


Figure 4.5 Average free amino acid concentration of the model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts: Ala (upper) and Leu (lower). The error bar in the figure represents the standard deviation of the repeated measurements of all samples; the number of the repeated measurements were 18 (n=18).

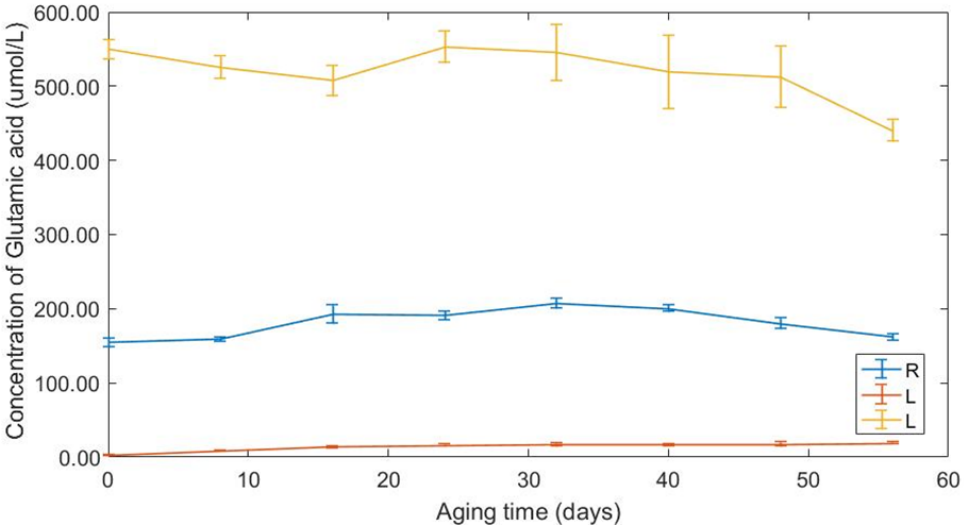


Figure 4.6 Average free amino acid concentration of the model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts: Glu. The error bar in the figure represents the standard deviation of the repeated measurements of all samples; the number of the repeated measurements were 18 (n=18).

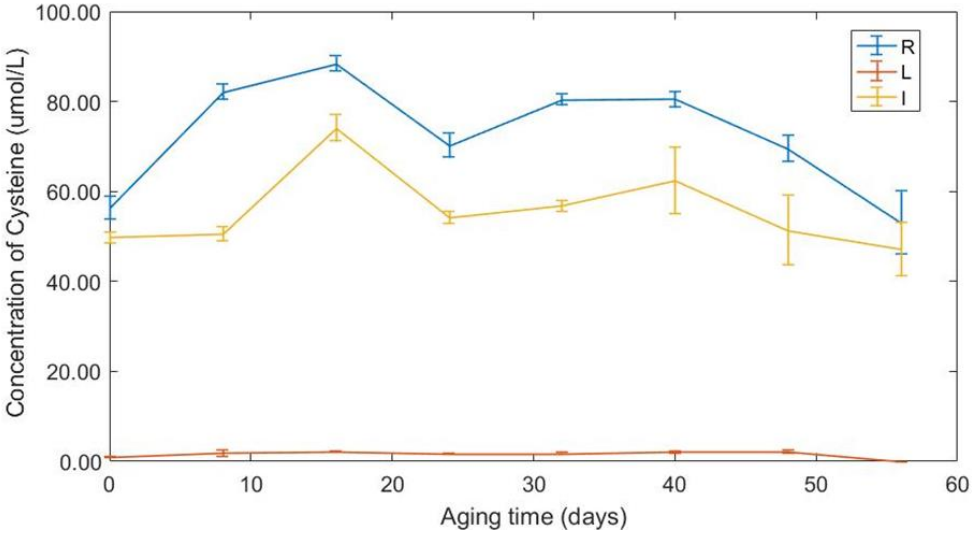


Figure 4.7 Average free amino acid concentration of the model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts: Cys. The error bar in the figure represents the standard deviation of the repeated measurements of all samples; the number of the repeated measurements were 18 (n=18).

Also of note were Ser and Thr because concentration changes for these two amino acids are considered evidence of yeast autolysis (Figures 4.8 and 4.9). The highest concentrations of Ser and Thr were found in samples obtained from treatment R, followed by I and L on day 56 (60, 40 and 10 µmol/L, respectively). The low concentrations for samples containing L this can be attributed to pre-

wash step. In more detail, Ser increased in model wines containing R and L, but was more stable in L In contrast, Thr increased in all samples. Increases in the concentrations of Ser and Thr had been attributed to the degradation of the mannoprotein released from yeasts cell walls (Van Der Vaart et al., 1995), and is consistent with findings of yeasts autolysis in model wine, sparkling wine, sparkling cider (Martínez-Rodríguez and Polo, 2000, 2001; Perrot et al., 2002; Guilloux-Benatier and Chassagne, 2003; Martínez-Rodríguez et al., 2002; Bozdoğan and Canbaş, 2012; Valles et al., 2005).

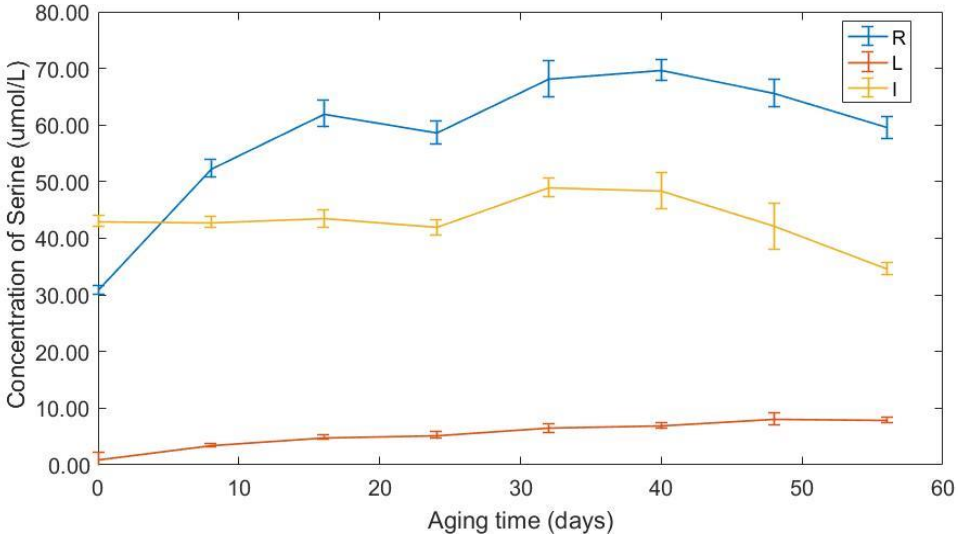


Figure 4.8 Average free amino acid concentration of the model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts: Ser. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n=18).

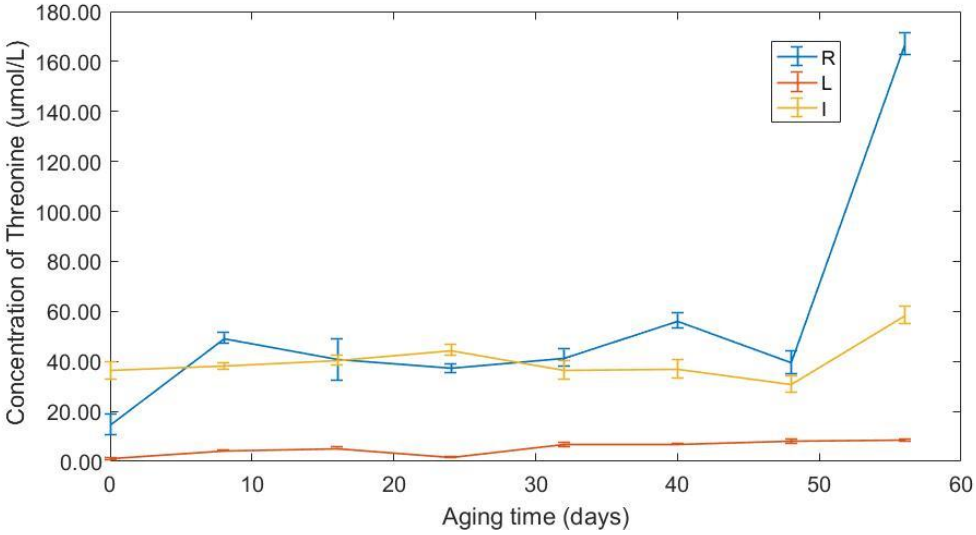


Figure 4.9 Average free amino acid concentration of the model wines containing rehydrated yeasts (R), collected lees (L) and inactivated yeasts: Thr. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n=18).

4.4.3 Polysaccharides

Neutral polysaccharides

The concentration of neutral polysaccharides in model wines containing different lees material was determined throughout an aging period of 160 days. Lees material, enzyme addition, stirring frequency, time and the interactions with time were all significant ($P < 0.05$) (Table 4.5). The interaction between enzyme addition and stirring frequency was not significant ($P > 0.05$). Although stirring frequency and its interaction with time and lees material was statistical significant, the magnitude of this effect was small compared with the interaction between time, enzyme addition and lees material.

Table 4.5 The results of ANOVA of neutral polysaccharides concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Enzyme	< 0.001
Lees material	< 0.001
Stirring frequency	0.018
Enzyme.Lees material	< 0.001
Enzyme.Stirring frequency	0.193
Lees material.Stirring frequency	< 0.001
Enzyme.Lees material.Stirring frequency	0.004
Block.Subject.Time stratum	
Time	< 0.001
Time.Enzyme	< 0.001
Time.Lees material	< 0.001
Time. Stirring frequency	0.003
Time.Enzyme.Lees material	< 0.001
Time.Enzyme.Stirring frequency	< 0.001
Time.Lees material.Stirring frequency	< 0.001
Time.Enzyme.Lees material.Stirring frequency	< 0.001

Figure 4.10 shows the change in neutral polysaccharides concentration in model wines containing different lees material throughout the aging experiment. Neutral polysaccharides were the major polysaccharides found in the model wine and constituted more than 90% of total polysaccharides throughout aging experiment for R and I, and more than 74% for L. Therefore, total polysaccharides followed a trend similar to that of neutral polysaccharides as also shown by other workers (Del Barrio-Galán et al., 2011). Thus, neutral polysaccharides concentration was used to represent total polysaccharide in the comparisons of results with previous studies.

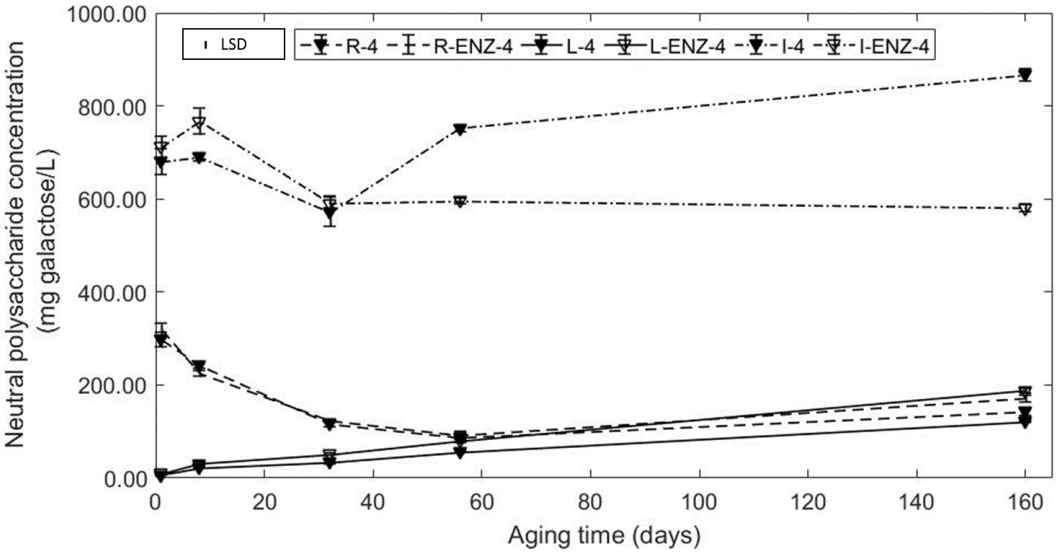


Figure 4.10 Neutral polysaccharide concentration in model wine containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 9).

Neutral polysaccharides concentration in treatment I varied over the aging period but remained at a higher level than in treatment R and L, and within the range of 600 to 800 mg galactose/L. Treatment R followed a trend whereby neutral polysaccharide concentration decreased from day 1 (300 mg galactose/L) and became stable from day 32 (approximately 200 mg galactose/L). In contrast, the neutral polysaccharide concentration of treatment L gradually increased throughout the aging experiment and reached a final concentration which was similar to treatment R.

The addition of enzyme significantly affected the release of polysaccharides from the different lees materials throughout the aging experiment ($P < 0.05$). The final neutral polysaccharide concentrations of R treatments with enzyme addition were greater than those without. In contrast, the opposite was observed for treatment I.

These differences can be attributed to the type of lees material. Inactivated yeasts are manufactured to quickly increase the concentration of polysaccharides. Del Barrio-Galán et al. (2011) found that neutral polysaccharides concentration of the wines containing yeast derivative products throughout an aging experiment were higher than wine containing lees. The polysaccharide concentrations of both lees and inactivated yeast treatments were lower in that study compared to the current study. This can be attributed to the lower dosages of lees (3% v/v wet fine lees) and yeast derivatives (0.05 to 0.4 g/L) used in that study (Del Barrio-Galán et al., 2011). Nunez et al. (2005) also studied autolysis of yeast in sparkling wine and found the yeast strain can affect polysaccharide content significantly ($P < 0.05$).

In a study of the trend in polysaccharide concentration over time, a slow but gradual increase of polysaccharide content (from 0.13 mg to 36. 83 mg pullulans/L during 9 months aging) was observed by Palomero et al. (2007). This is similar to treatment L in the current study. Other authors (del Barrio-Galán et al., 2015; Del Barrio-Galán et al., 2012; Loira et al., 2013) evaluated the aging of wines on lees with different yeast strains, oak chips and several yeast derivative products (containing high levels mannoprotein) and found all the applied techniques were able to increase the release of total and neutral polysaccharides. A decrease of total and neutral polysaccharide content was also demonstrated by other authors (Guadalupe & Ayestarán, 2007) (Del Barrio-Galán et al., 2011; Martínez-Lapuente et al., 2013; Rodriguez-Nogales et al., 2012), similar to that observed in treatment R in the current study. Pati et al. (2010) suggested that β-glucanase is able to reduce polysaccharide molecular weight, change their composition, and modify their hydrophobic properties and consequently their solubilisation. It has been suggested that total and neutral polysaccharide concentration would decrease when the precipitation rate of the released polysaccharides became higher than their solubilisation rate (Martínez-Lapuente et al., 2013). Studies on yeast derivatives found that the neutral polysaccharide concentration were decreased from 100-140 mg/L at the beginning of aging to 70-90 mg/L at 6 months of aging. Therefore, the precipitation of polysaccharide might be the reason for the lower neutral polysaccharides concentration in treatment I with enzyme addition from day 32. Differences in treatment I toward to the end of aging might be attributed to the 'quick release' feature of inactivated yeasts which released more polysaccharides without enzyme addition; these quickly released polysaccharides can be further breakdown rapidly in the presence of β-glucanase. Palomero et al. (2007) reported a rapid breakdown of the yeast cell wall in the model with the addition of β-glucanase; the fragments from polysaccharide breakdown were found to be usually smaller and more uniform in size. These smaller fragments might be more susceptible to precipitation.

Palomero et al. (2007) also carried out work on autolysis of different yeast strains in the presence of β-glucanase during aging on lees in model wine; the study found that the addition of the enzyme can significantly accelerate yeast autolysis. For example, polysaccharides released by autolysis from yeasts in the presence of β-glucanase increased from about 2.7 mg pullulans/L at week 1 to 57.2 mg pullulans/L at week 3 which is much higher than conventional autolysis after 9 months of aging (36.83 mg pullulans/L) (Palomero et al., 2007). These results were consistent with those from treatments R and L for which higher neutral polysaccharides concentrations were found in the model wine in the presence of β-glucanase throughout aging.

Acidic polysaccharides

The concentration of acidic polysaccharides in model wines containing different lees material was also determined throughout an aging period of 160 days. Overall, lees material, enzyme, stirring frequency, time and their interactions all had a significant effect ($P < 0.05$) (Table 4.6). There were no significant interaction between the enzyme and stirring frequency, and the interaction between enzyme, lees material and stirring frequency ($P > 0.05$).

Figure 4.11 shows the acidic polysaccharide concentration of model wine containing different lees material throughout aging experiment. In general, the acidic polysaccharide concentration in all treatments were low (< 10 mg galacturonic acid/L) throughout the aging experiment, consistent with previous studies with treatments containing lees (3% v/v) and yeast derivatives (< 15 mg/L) (Del Barrio-Galán et al., 2011).

Mannoprotein

A subset of samples (treatments with stirring frequency of every 4 days) was chosen to examine the differences between mannoprotein concentration due to the lees material, enzyme addition, and time factors. Overall, lees material, enzyme, time and their interactions all had a significant impact on the concentration of mannoproteins in model wine ($P < 0.05$) (Table 4.7).

Table 4.6 The results of ANOVA of acidic polysaccharide concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Enzyme	< 0.001
Lees material	< 0.001
Stirring frequency	0.786
Enzyme.Lees material	< 0.001
Enzyme.Stirring frequency	0.353
Lees material.Stirring frequency	< 0.001
Enzyme.Lees material.Stirring frequency	0.558
Block.Subject.Time stratum	
Time	< 0.001
Time.Enzyme	< 0.001
Time.Lees material	< 0.001
Time.Stirring frequency	< 0.001
Time.Enzyme.Lees material	< 0.001
Time. Enzyme.Stirring frequency	0.047
Time Lees material.Stirring frequency	< 0.001
Time.Enzyme.Lees material.Stirring frequency	0.012

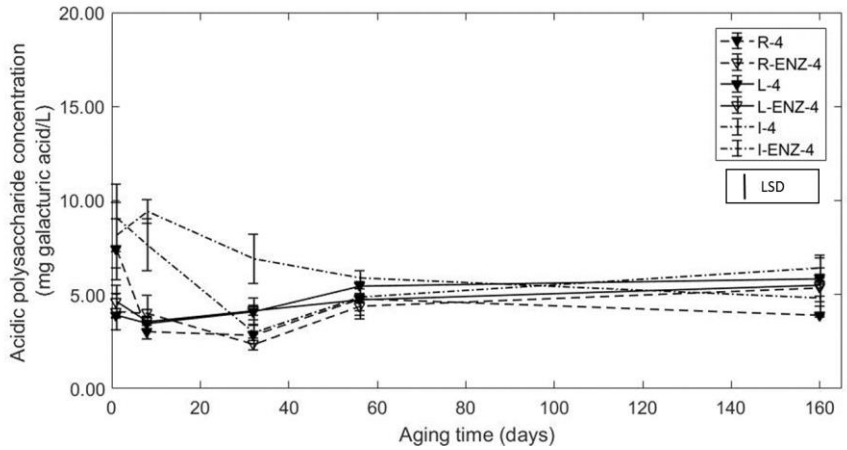


Figure 4.11 Acidic polysaccharide concentration in model wine containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 9).

Table 4.7 The results of ANOVA of the concentration of mannoprotein in model wines.

Source of variation	F probability
Block.Subject stratum	
Lees material	< 0.001
Enzyme	< 0.001
Enzyme.Lees material	< 0.001
Block.Subject.Time stratum	
Time	< 0.001
Time.Lees material	< 0.001
Time.Enzyme	< 0.001
Time.Enzyme.Lees material	< 0.001

Figure 4.12 shows the changing concentration of mannoprotein in the model wine containing different lees material during yeast autolysis. At day 56, the highest concentration of mannoprotein was found in the samples containing inactivated yeasts (I) (80 to 160 mg/L), followed by rehydrated yeasts (R) (25 to 30 mg/L), and collected lees (L) (10 to 20 mg/L). Aging time affected the concentration of mannoprotein in all model wines. The increasing trends were observed in the model wine containing I (approximately 50 mg/L at day 1 to 80 mg/L at day 56), R (from 5 to 30 mg/L), and L (from 5 to 20 mg/L) (Figure 4.12). The impact of aging time on concentration of mannoprotein was also reported by Pérez-Magariño et al. (2015), Juega et al. (2015), Martínez-Lapuente et al. (2013) and Dupin et al. (2000) from dry yeast products and *Saccharomyces cerevisiae* into the model wine, sparkling wines, and Albariño white wine. For example, Dupin et al. (2000) demonstrated the changes during storage on yeast lees (yeasts propagated in chemically defined grape juice medium), and found that the concentration of polymeric mannose was increased from 0 mg/L to 15 mg/L at week 8, similar to the results in sample containing collected lees (L) in the current study.

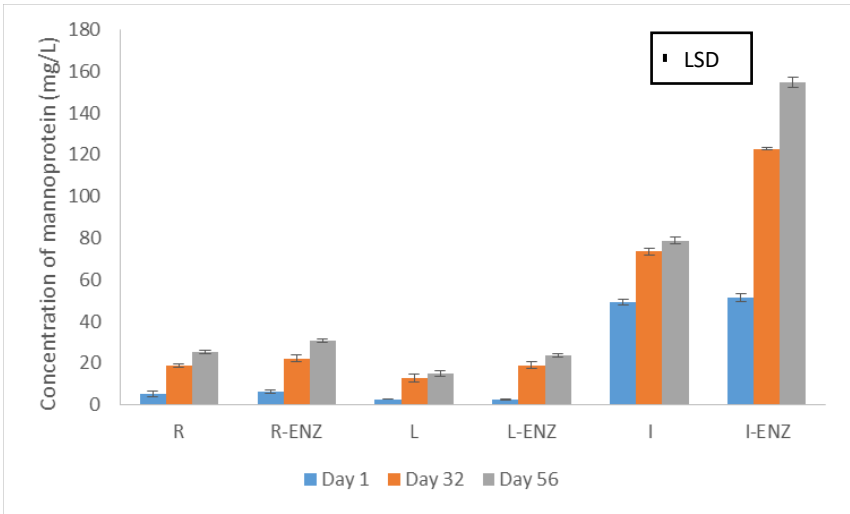


Figure 4.12 Mannoprotein concentration in model wine containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 3).

Different amounts of mannoprotein was released from different lees materials, R, L and I. For example, on day 1, the highest concentration of the mannoprotein was found in the model wine containing I (50 mg/L). Such observation can be attributed to two reasons. Firstly, the strain used for manufacturing the inactivated yeasts might contain more soluble mannoproteins than rehydrated yeasts (EC1118) and collected lees. This was consistent with the finding reported by Pérez-Magariño et al. (2015)and Del Barrio-Galán et al. (2011). Del Barrio-Galán et al. (2011) compared the release of mannoprotein from lees and three different dry yeast derivative products during white wine aging, and the concentration of mannoprotein was higher in the wine treated with yeast derivative products (ranged from 74 to 113 mg/L) than the one aged on lees (60 mg/L) after 6 months. Secondly, the damage to cell walls during the inactivation process facilitates a more rapid release of mannoprotein, and possibly increased surface area of cell wall for hydrolysis by β-glucanase.

Addition of β-glucanase into the model wines increased the release of mannoprotein during the aging experiment. On day 56, the concentration of model wines treated with β-glucanase all had a higher concentration of mannoprotein than the untreated ones. There is limited published information on the effect of β-glucanase addition on the concentration of mannoprotein during aging. However, the findings described by Torresi et al. (2014) and Palomero et al. (2007) indirectly demonstrated the effects of β-glucanase on releases of mannoprotein. For example, Palomero et al. (2007) investigated the release of polysaccharides from yeast cell walls during conventional and enzyme-assisted autolysis during aging and found that in just two weeks, the amounts of polysaccharides (ranged from 26 to 69 mg pullulans/L) were similar to or even higher than those produced in nine months by the conventional method (22 to 37 mg pullulans/L).

β-glucan

Analysis of variance was also used to examine differences in the concentrations of β-glucan due to the lees material, enzyme addition, and time factors. Overall, lees material, enzyme addition, time and their interactions (except that between lees material and enzyme addition) all had a significant impact on the concentration of β-glucan in model wine ($P < 0.05$) (Table 4.8). Aging time and lees material were the two main factors that affected the concentration of β-glucan. The effect of enzyme addition was small; the grand mean of the concentrations of β-glucan in the model wines with and without enzyme addition were 8.74 and 9.66 mg/L, respectively.

Table 4.8 The results of ANOVA of the concentration of β-glucan in model wines.

Source of variation	F probability
Block.Subject stratum	
Lees material	< 0.001
Enzyme	0.008
Enzyme.Lees material	0.181
Block.Subject.Time stratum	
Time	< 0.001
Time.Lees material	< 0.001
Time.Enzyme	0.009
Time.Enzyme.Lees material	0.031

The β-glucan concentration in model wine containing different lees materials during aging is shown in Figure 4.13. Martínez-Lapuente et al. (2013) found that the amount of β-glucan released during sparkling wine production was affected by aging time. During 30 months of on-lees aging in bottles, the concentration of β-glucan increased from the month 3 to 9 (i.e. 45 to 85 mg/L in sparkling made from Verdejo grape), followed by a decrease from month 9 to month 30 (i.e. 85 to 14 mg/L). In contrast, a decrease of β-glucan concentration was not observed in the current study. This might be attributed to the application of periodic stirring because lack of stirring of lees in sparkling wine caused a reduction of hydrolytic enzyme activities involved in the autolytic process and a lower release of yeast polysaccharides (Martínez-Lapuente et al., 2013).

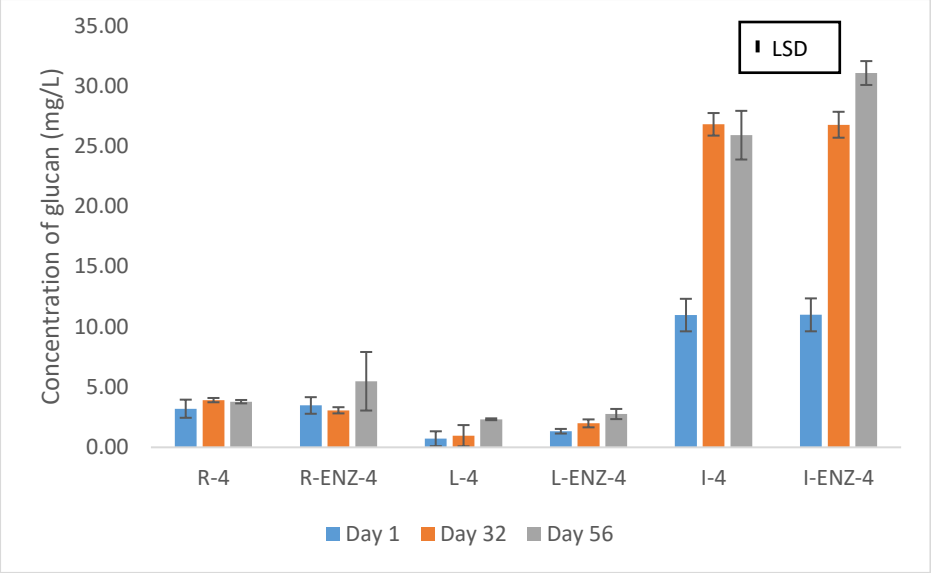


Figure 4.13 The β-glucan concentration in model wine containing different lees materials: rehydrated yeast (R), collected lees (L) and inactivated dry yeast (I), stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 3).

Lees material affected the concentration of β-glucan in all model wines. The highest β-glucan concentration was observed in the samples containing inactivated yeasts, followed by rehydrated yeasts and collected lees with grand means of 22.09, 3.82 and 1.68 mg/L, respectively. This observation can be explained by similar reasoning to that described for mannoprotein. There are limited publications investigated the difference of β-glucan concentration in the model wine containing different lees materials. Thus, the current study provides new information.

Mannoprotein-to-β-glucan ratio

Mannose and glucose were only two sugar monomers identified in the breakdown products of extracted polysaccharides (Figure 4.14). Galactose was not found in any treatment, and if present was at concentrations below the detection limit of the HPLC method. This result indicated that macromolecules released from the different lees materials used in the current study were composed of mannoprotein and β-glucan. On day 56 of the aging experiment, mannose and glucose comprised 75-90% and 12-25% of the sugars, respectively. This was similar to Rodrígues et al. (2012) who analysed the 160 kDa fraction from wine.

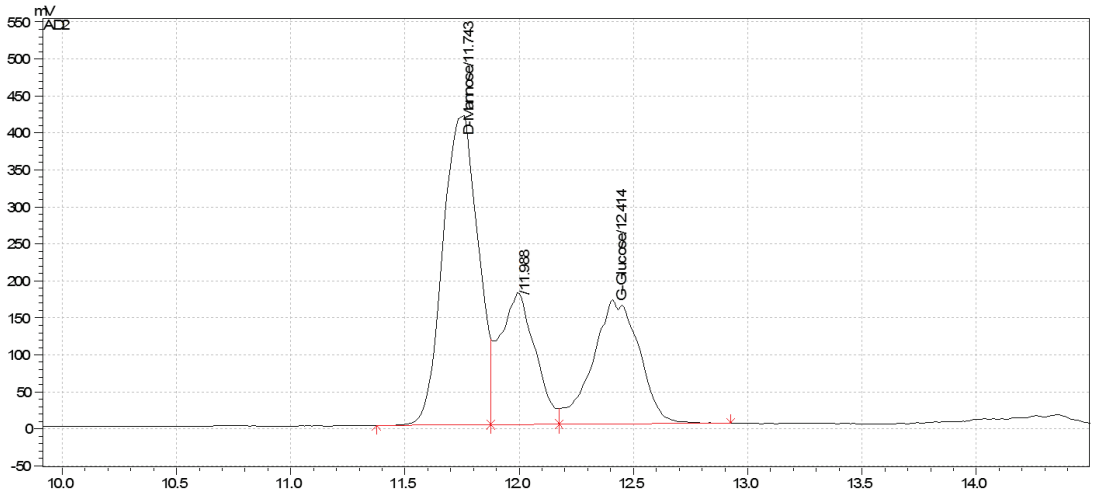


Figure 4.14 Example of chromatogram of the monosaccharide after extraction and hydrolysis of polysaccharide from treatment R, L, and I.

In the model wine containing the rehydrated yeasts and collected lees, the ratio of mannose/glucose of the samples without enzyme addition increased during aging (Table 4.9). This is consistent with the previous study of the sparkling wine aging, in which an increase mannose/glucose ratio was found from 18 to 30 months of aging (Martínez-Lapuente et al., 2013). It has been suggested that the increase in the mannose/glucose ratio during yeast autolysis was because β-glucan is more susceptible to precipitation than mannoproteins (Martínez-Lapuente et al., 2013). In this study for model wine samples with enzyme addition, an increase of ratio of mannose/glucose was only observed during the first 32 days, and a decrease of the mannose/glucose ratio was found toward the end of the aging experiment. This was expected because in the presence of β-glucanase, the release of β-glucan from yeast cell walls and the rate of the further breakdown are greater than without β-glucanase. The results are consistent with this hypothesis.

Table 4.9 The mannose/glucose ratio in the model wine containing different lees material during the aging experiment.

Sample	Day 1	Day 32	Day 56	% of the total sugar at Day 56	
				Mannose	Glucose
R-4	1.67	4.79	4.97	86.86	13.14
R-ENZ-4	1.84	7.24	5.48	88.34	11.66
L-4	n.d	n.d	5.48	86.50	13.50
L-ENZ-4	1.86	9.76	6.94	89.57	10.43
I-4	4.53	2.74	2.85	75.27	24.73
I-ENZ-4	4.71	4.59	3.95	83.29	16.71

n.d: not detected due to below the detection limit of HPLC-ELSD.

In the model wine containing inactivated yeast, the ratio of mannose/glucose of the sample without enzyme addition decreased during the first 32 days followed by a slight increase. As the cell walls of inactivated yeasts were already partially disrupted, an earlier release of β-glucan was expected. In the samples with enzyme addition, only a slight decrease of mannose/glucose ratio was observed throughout the aging experiment. This might be attributed to that in the presence of β-glucanase, the release rate of β-glucans from yeast cell walls was greater than without the enzyme addition.

4.4.4 Glyerol

The glycerol content of the model wine containing different lees material was determined throughout an aging period of 56 days. Overall, there were significant effects due to lees material, enzyme addition, time and the interaction between these factors ($P < 0.05$), but not from stirring frequency ($P > 0.05$) (Table 4.10).

Table 4.10 The results of ANOVA of glycerol concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Enzyme	< 0.001
Lees material	< 0.001
Stirring frequency	0.128
Enzyme.Lees material	< 0.001
Enzyme.Stirring frequency	0.266
Lees material.Stirring frequency	0.229
Enzyme.Lees material.Stirring frequency	0.366
Block.Subject.Time stratum	
Time	< 0.001
Time.Enzyme	< 0.001
Time.Lees material	< 0.001
Time.Stirring frequency	0.019
Time.Enzyme.Lees material	< 0.001
Time. Enzyme.Stirring frequency	0.002
Time Lees material.Stirring frequency	< 0.001
Time.Enzyme.Lees material.Stirring frequency	0.060

Figure 4.15 shows the glycerol content of model wines containing rehydrated yeasts throughout aging experiment. In general, a sharp increase in glycerol content from 18 to 28 mg/L was observed in the first eight days; then the increase of glycerol content slowed down and reached the peak on day 24, then became stable throughout the rest of the aging experiment. The glycerol content in the model wine containing inactivated yeasts was much lower which ranged from 6 to 13 mg/L, approximately; and increase of glycerol content in the model wine containing inactivated yeasts was only observed in the wine with presence of enzyme (Figure 4.16). The glycerol content in the model wine containing collected yeasts was the lowest among three lees material. It was less than 3.9 mg/L which was the detection limit of the analytical technique.

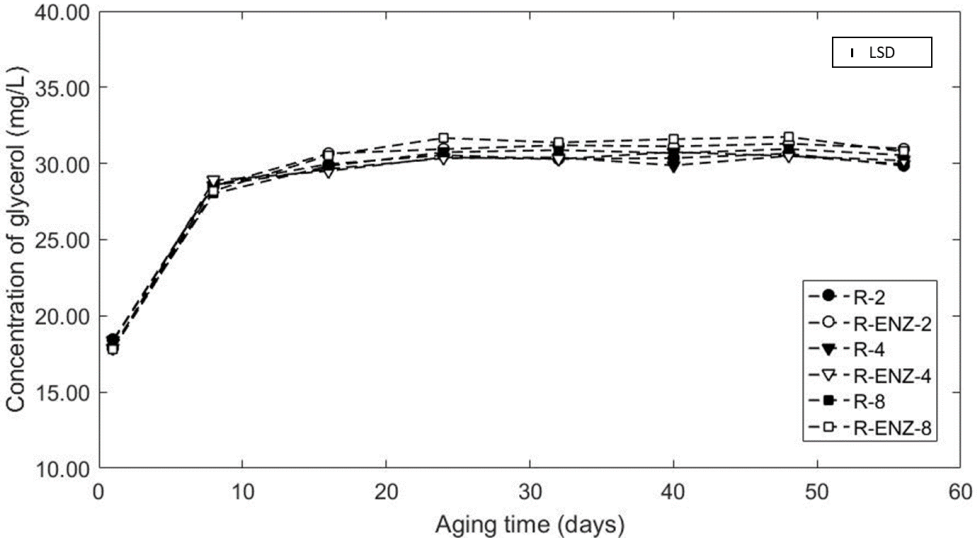


Figure 4.15 The glycerol concentration of model wines containing rehydrated yeasts (R): stirred every2, 4 and 8 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 3).

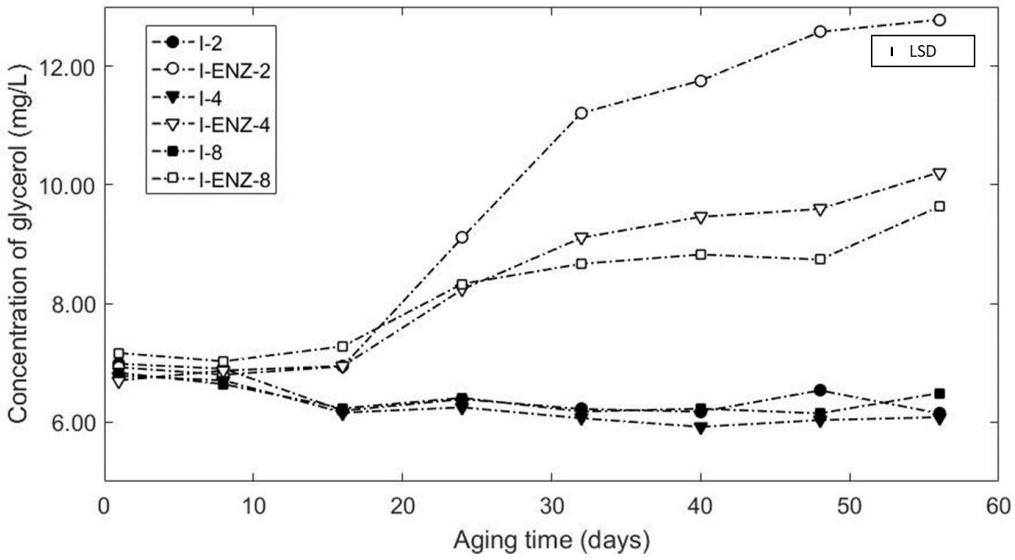


Figure 4.16 The glycerol concentration of model wines containing inactivated dry yeasts (I): stirred every 2, 4 and 8 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 3).

Growing yeast cells produce and accumulate glycerol to balance intracellular osmolality to prevent dehydration (Aslankoohi et al., 2015). This accumulated glycerol can be released into the surrounding medium as was found in the model wines containing rehydrated yeasts. The lower glycerol content in the model wines containing inactivated yeasts was because the inactivation process destroys the viability of yeast cells. Similarly, the low glycerol content in the model wine containing collected lees was expected; since the lees was stored in wine and was pre-washed with

0.9% NaCl (v/v) before addition.

The addition of enzyme significantly affected the released of glycerol content from different lees material throughout the aging experiment, especially in the model wine containing inactivated yeasts ($P < 0.05$). In the model wines with no enzyme addition, there was no significant changing of glycerol content throughout the whole aging experiment. In contrast, commencing on day 16 there was a steady increase of glycerol content in the model wine with enzyme addition. This can be attributed to enzymatic break down of yeast cell membranes and release of glycerol to the model wine.

Although the effect of stirring frequency was not statistically significant, there was a significant interaction between stirring frequency, enzyme addition and time ($P < 0.05$). In the model wine containing inactivated yeasts, a more rapid increase of glycerol content was found with more frequent stirring frequency with the presence of the enzyme. For example, the most rapid increase of glycerol content was observed in I-ENZ-2, followed by I-ENZ-4 and I-ENZ-8. Thus, higher stirring frequency increased the extraction of glycerol from yeast cells.

In general, even though the glycerol content can be increased by manipulating lees according to the current study, the effects on mouthfeel of wine can be very limited. As a component of wine, glycerol usually ranges from 4 to 16 g/L (e.g. Sauvignon blanc 6.31 g/L; Shiraz, 10.22 g/L; Cabernet Sauvignon, 10.81 g/L) (Calderone et al., 2004; Nieuwoudt et al., 2017). Gawel et al. (2007) studied the effects of ethanol and glycerol on body of Riesling wine and suggested the difference in glycerol content can affect perceived viscosity. However, the reported difference in glycerol content was 5 g/L. Therefore, the addition of c. 30 mg/L of glycerol during lees manipulation is unlikely to be effective in increasing the viscosity of the aged wine. In the more recent sensory study, Runnebaum et al. (2011) indicated that ethanol and glycerol do not significantly contribute to the viscous mouthfeel.

4.4.5 Viscosity

The viscosity of the model wine containing different lees material was determined throughout an aging period of 160 days. There were no effects of the lees material, enzyme, stirring frequency, time and their interaction on viscosity in model wines ($P > 0.05$) (Table 4.11).

Table 4.11 The results of ANOVA of viscosity in model wines.

Source of variation	F probability
Block.Subject stratum	
Enzyme	0.803
Lees material	0.143
Stirring frequency	0.116
Enzyme.Lees material	0.230
Enzyme.Stirring frequency	0.162
Lees material.Stirring frequency	0.387
Enzyme.Lees material.Stirring frequency	0.511
Block.Subject.Time stratum	
Time	0.045
Time.Enzyme	0.139
Time.Lees material	0.359
Time.Stirring frequency	0.455
Time.Enzyme.Lees material	0.558
Time.Enzyme.Stirring frequency	0.809
Time Lees material.Stirring frequency	0.075
Time.Enzyme.Lees material.Stirring frequency	0.219

In the current study, the viscosity of the model wine containing different lees material ranged from 1.52 to 1.86 mPa.s. (Figure 4.17). Yanniotis et al. (2007) determined viscosity of commercial red and white dry and sweet wines by using falling ball viscometer and determined values of 1.71 to 1.80 mPa.s for dry white, 1.88 to 1.92 mPa.s for dry red wine, and 3.04 to 3.16 for sweet wine. The lower values found for model wines in the current study can be attributed to differences in alcohol, dry extract and glycerol, alcohol content and dry extract being identified as two major factors that influence wine viscosity (Neto et al., 2015; Yanniotis et al., 2007). The alcohol content of the model wine used in the current study was 10% v/v which is lower than the reported commercial wine (ranged from 11.6 to 12.7 % v/v). The dry extract content of model wine must be lower than the commercial wine due to its simpler, reduced composition compared to commercial wine. Dang (2013) found that alcohol and glycerol were highly correlated with viscosity.

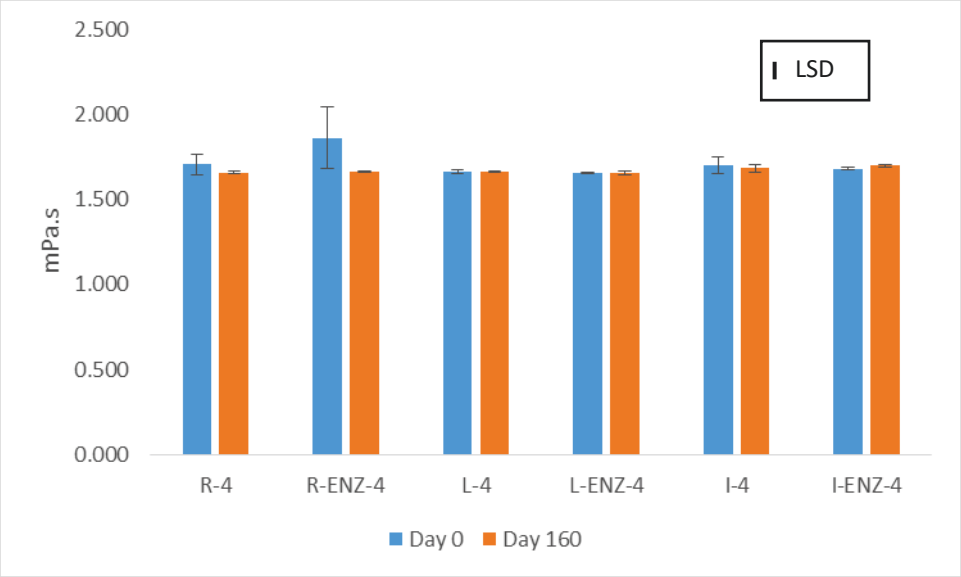


Figure 4.17 The viscosity of the model wines containing different lees materials: rehydrated yeasts (R), collected lees (L) and inactivated dry yeasts (I): stirred every 4 days, without and with addition enzyme (ENZ). The black bar in the figure represents the least significant difference (LSD) (n = 18).

4.5 General discussion and conclusions

Principal component analysis (PCA) was undertaken in order to investigate the overall combined effects of the different lees treatment [lees material (inactivated yeasts, rehydrated yeasts and collected lees), stirring frequency (every 2, 4 and 8 days), and enzyme addition (β-glucanase: 0U and 7.5U)] on the composition model wines after 8 weeks of on-lees aging (Figure 4.18). The first two principle components explained 88% of the variance in the dataset.

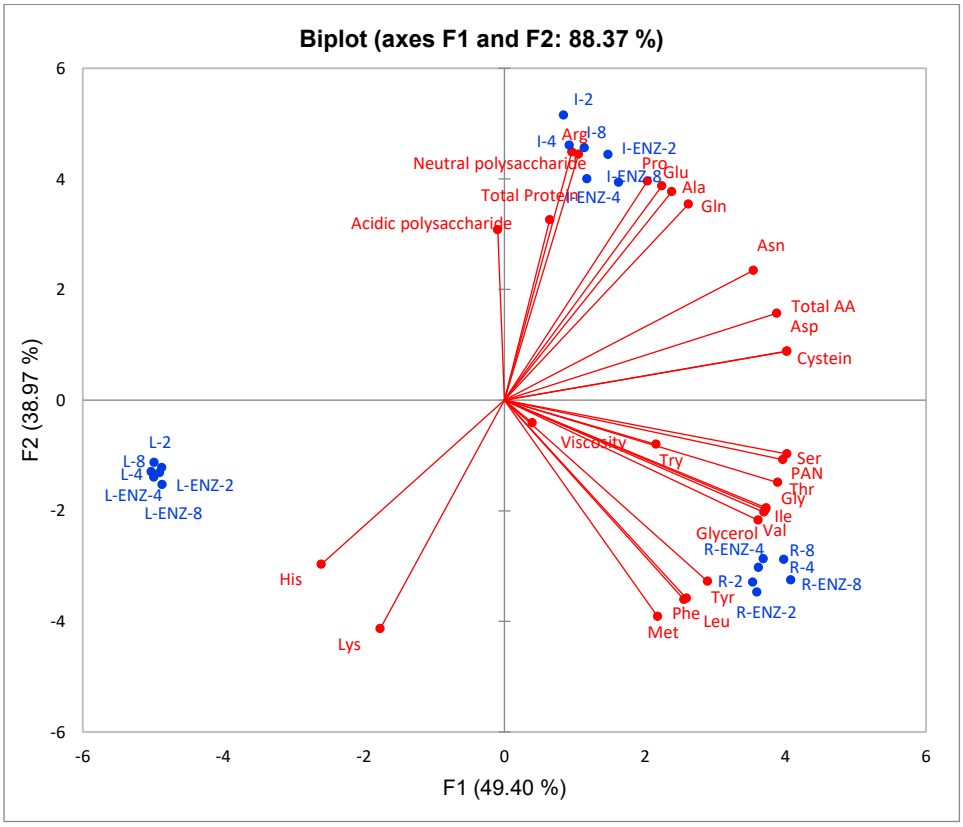


Figure 4.18 Principle component analysis (PCA) of the effect of lees treatments on the different chemical compounds released into the model wines.

This analysis clearly demonstrated the importance of the source of the lees material on the overall composition of model wines. All samples from model wines containing collected lees had a negative value for F1 and positive value for F2 (Figure 4.18). The concentrations of most chemical compounds in samples containing collected lees were below the average for the trial, the exceptions being His and Lys. Within this group different treatments were poorly separated, which indicated that stirring frequency and enzyme addition had minor impact on composition. This was most likely because a high proportion of soluble potentially soluble compounds had been removed as a result of previous use, storage and the pre-wash step.

All samples from the model wine containing rehydrated (R) and inactivated (I) yeasts had a positive value for F1, and either positive (for I) or negative (for R) value for F2. This clearly showed that, on

average, the samples from model wine containing rehydrated yeasts had concentrations of certain amino acids (including Met, Phe, Leu, Tyr, Ile, Gly, Val, Thr, and Ser), glycerol and primary amino nitrogen that were above average; the samples from model wine containing inactivated yeasts had concentrations of the certain amino acids (including Arg, Pro Glu, and Ala), total protein and neutral polysaccharides that were above average. This can be attributed to the difference between chemical compositions of the lees materials. In addition, within the same lees material, the different treatments were well separated, which indicated that the lees materials and their interaction with stirring frequency and the enzyme addition had significant impacts on those measured chemical compounds stated above. However, the stirring frequency and enzyme addition were minor factors compare to lees material. Figure 4.18 also demonstrated the correlations between the different chemical compounds. Interestingly, the total protein content was correlated to neutral polysaccharide content. This might suggest that the mannoprotein released during on-lees aging contributed to neutral polysaccharide content. This is consistent with the results described by Gonçalves et al. (2002) who showed that 32% of the total polysaccharide content in the studied white wine corresponded to mannoproteins.

Principle component analysis (PCA) was also performed only on the smaller set samples in which mannoprotein content was determined (Figure 4.19), and the results showed similarity to Figure 4.18. This also showed that, on average, the samples from model wine containing inactivated yeasts had concentrations of mannoprotein above average. In addition, the biplot showed that, total protein, mannoprotein, β-glucan, Arg and neutral polysaccharide were highly correlated. This supports the suggested contribution of the mannoprotein to neutral polysaccharide content in the model wine. Interestingly, viscosity was found correlated to the total protein, mannoprotein, β-glucan, Arg and neutral polysaccharide. Gawel et al. (2016) demonstrated that a medium molecular mass polysaccharide (13-93 kDa) containing mannoprotein reduced palate hotness and increased viscosity at higher pH. In addition, Ser and Thr were show not to be correlated to mannoprotein. This suggested that the appearance of Ser and Thr during the on-lees aging might indicate the onset of yeast autolysis, and may not be a suitable marker for predicting the final concentration of mannoprotein.

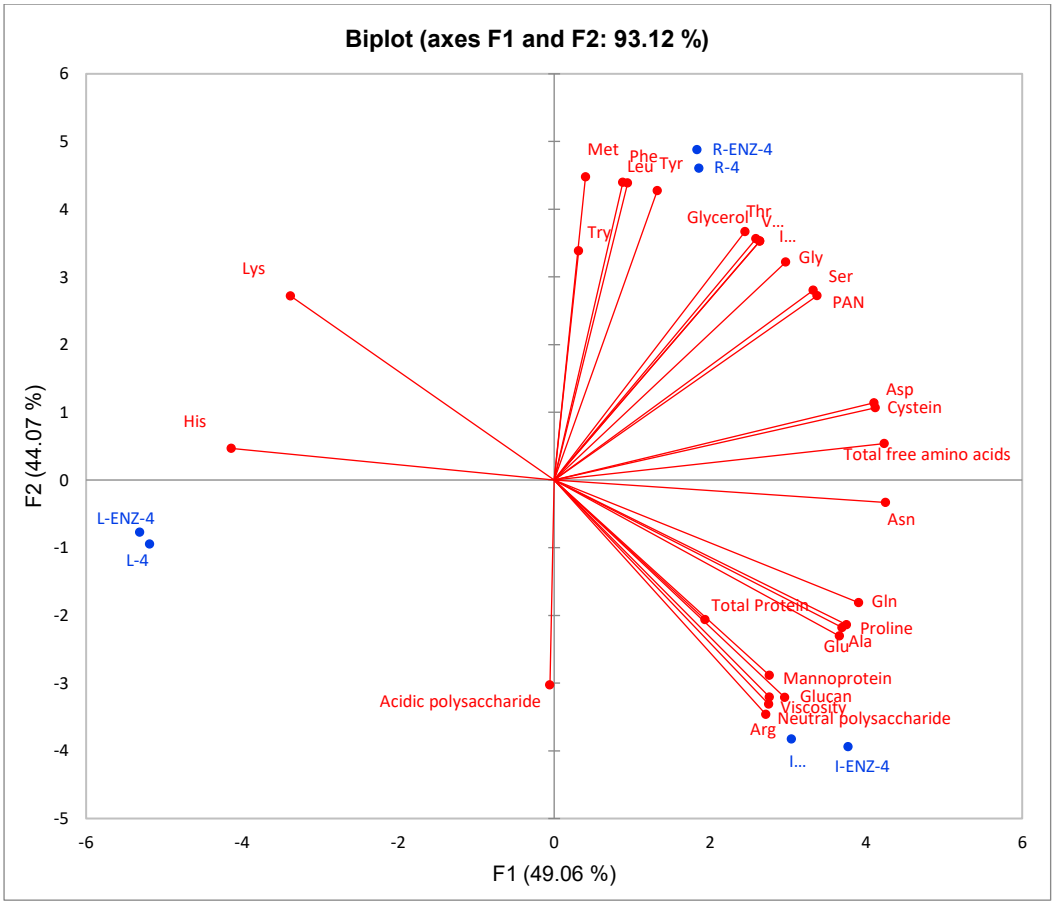


Figure 4.19 Principle component analysis (PCA) of the effect of lees treatments (stirring every 4 days and addition of β-glucanase) on the different chemical compounds released into the model wines.

A radar chart was used to demonstrate the effectiveness of lees treatments for release of components (total protein, neutral polysaccharide, mannoprotein, β-glucan, Arg, Glu, Pro, and Ala) that have been highlighted as contributing to improved wine mouthfeel (Figure 4.20). To avoid differences due to different concentrations between chemical compounds, results were normalized against the average of each compound. The areas of each lees material represent the effectiveness of the lees treatments at the end of aging experiment. The application of inactivated yeasts (I-4 and I-ENZ-4) should be the most effective way to improve wine mouthfeel due to their ability of release of a larger amount of neutral polysaccharide, mannoprotein and β-glucan than rehydrated yeast and collected lees. Inactivated yeasts also might be the best lees material for improving umami taste compared with rehydrated yeasts and collected lees. Addition of β-glucanases increased the release of mannoprotein and β-glucan from all lees materials. For example, the mannoprotein concentration in treatment I-ENZ-4 increased two-fold compared with I-4. However, this also lead to decreases of the concentration of total protein and glutamic acid. This might be due to the β-glucanases addition enhancing the release of protease from lees materials.

Figure 4.20 The example of the effectiveness of lees treatments (stirring every 4 days) on the release of the chemical compounds which may potentially improve wine mouthfeel.

In general, total protein, neutral polysaccharide, mannoprotein, β-glucan were the major chemical compounds which correlated to wine viscosity. Thus, these compounds might be the ones that made a contribution to wine mouthfeel. Lees material was the major factor affecting the release of the above chemical compounds, and inactivated yeasts were the best option followed by rehydrated yeasts and collected lees. Application of stirring frequency of every 4 days combined with an addition of β-glucanase should be the best way for accelerating the aging process.

Chapter 5

Effect of Pulsed Electric Field on Chemical Components Released by Three Different Yeasts after Aging on Lees in a Model Wine

5.1 Introduction

On-lees (sur lies) aging is a traditional method for the elaboration of white wines (Tao et al., 2014). In the previous chapter, total protein, neutral polysaccharide, mannoprotein and β-glucan concentration were found to be significantly affected by Lees material; and application of stirring frequency of every 4 days combined with an addition of β-glucanase should be the best way for accelerating aging. Even though conventional vinification techniques (e.g. periodic stirring and addition of β-glucanase) have been applied by the industry, on-lees aging is still regarded as a time-consuming process. This is due to the slow progress of yeast autolysis at the low aging temperature (lower than 10°C) and wine pH (~ 3.5), at which the activity of the enzymes is limited. Induction of yeast autolysis can be the key to accelerate the progress of on-lees aging.

Pulsed electric field (PEF) is a novel non-thermal processing technique which delivers microsecond high voltage pulses to a material placed between two electrodes (Puétolas et al., 2010). This generates pores in yeast cell membrane, causes cell death and the release of cellular contents. For example, Puértolas et al. (2010) showed that the PEF treatment can be used to inactivate bacteria and yeast by rupturing the cell structure. Recently, Martínez et al. (2016) demonstrated the potential of the application of PEF to induce accelerated autolysis of yeasts during on-lees aging. For example, the concentration of mannoprotein in the supernatant containing PEF treated (15 and 45 kV/cm for 45 and 150 μs) yeast cells of *Saccharomyces cerevisiae* was found to be significantly higher than the control samples after 18 days of incubation (Martínez et al., 2016). However, the release of nitrogenous compounds, polysaccharides, mannoprotein, β-glucan, and glycerol from the yeast as a result of induced autolysis remain poorly investigated. In addition, the acceleration of yeast autolysis and the impact of this process on the organoleptic quality of wine has not been investigated and their beneficial or negative effects are still to be defined.

The objectives of this study were to investigate the release of chemical compounds from PEF induced yeast autolysis using rehydrated active yeast; provide a general understanding of the release mechanisms with different PEF treatment strength and the application of the different enzymes levels; and to compare the effectiveness of PEF technology with conventional aging methods.

5.2 Experimental design

The experimental work in this chapter consisted of two parts: Experiment I and II. Experiment I aimed to optimize the processing parameters of the PEF treatment (Table 5.1). In experiment II, the release of chemical compounds from different lees types (inactivated yeasts, rehydrated yeasts and PEF-treated) was investigated.

Table 5.1 The experimental design of the optimization of operational parameters of PEF treatment.

Run	Strength (kV/cm)	Time (μs)	Frequency (Hz)
1	5.5	1	5
2	1	15.5	5
3	5.5	15.5	3
4	5.5	15.5	3
5	1	1	3
6	5.5	30	5
7	1	30	3
8	5.5	15.5	3
9	5.5	15.5	3
10	10	15.5	1
11	5.5	30	1
12	1	15.5	1
13	10	15.5	5
14	10	30	3
15	5.5	15.5	3
16	10	1	3
17	5.5	1	1

Figure 5.1 DIL pilot-scale pulsed electric field equipment.

5.2.1 Experiment I

Effect of the washing step and temperature

Active dry yeasts (AWRI R2) were rehydrated in deionized water (DI) according to the manufacturer's instruction before being added to a model wine. The rehydrated yeasts were then divided into three parts, F1, F2 and F3 and stored in 200 mL centrifuge bottles. F1 and F2 were centrifuged at 2000 rpm for 5 minutes, and the supernatant was then discarded. DI water and 0.9% (v/v) NaCl solution was added to F1 and F2, respectively. The ratio between rehydrated yeasts and DI water or 0.9% (v/v)

NaCl were 1:1 (w/w). The rehydrated yeasts in F1 and F2 were well suspended before centrifugation and the supernatant which resulted from the centrifugation was discarded. The washing step was repeated three times, and the washed rehydrated yeasts of F1 and F2 were re-suspended in DI and 0.9% (v/v) NaCl solution at a solid to liquid ratio of 1:1 (w/w). Subsamples of F1 and F2 were then incubated at 10, 15, 20, 25 and 30°C for 1 hour. The absorbance of the samples at 600 nm (OD 600) and the absorbance of the sample supernatants were measured at 260 nm and 280 nm as described in Section [3.2.1].

Effect of PEF parameters

Response surface method (RSM) was used to optimise the processing parameters of PEF treatment. Design Expert version 8.0.1 (Stat-Ease, USA) was used to create RSM design (Table 5.1). Before PEF treatment, the rehydrated yeasts were washed three times as described in Section 5.2.1, and stored on ice until use. The PEF system used in this investigation was Elcrack-HPV5, (DIL, Quakenbruck, Germany) (Figure 5.1). The PEF system was used in batch mode and the treatment chamber used had the following dimensions 13×8×5 cm. An oscilloscope (model UT2025C, Uni-Trend Group Ltd, Hong Kong, China) was used to monitor the pulse shape used. The rehydrated yeast suspension was poured into the treatment chamber until it was full. The processing parameters of PEF treatment were set according to Table 5.1. The cells were subjected to bipolar square waveform pulses of 50 to 300 µs of electric field strengths between 5.5 to 10 kV/cm at room temperature and applied at a frequency ranged between 20 and 100 Hz. The quantification of the number of the electroporated cells was determined by P.I staining method as described in Section 3.7. The OD600 of the sample, and the 260 nm and 280 nm of the sample supernatant were then monitored as described in Section 3.2.4.

5.2.2 Experiment II

A factorial study incorporated four different lees type [PL (rehydrated yeasts treated by low level PEF strength, 5.5 kV/cm, 16 µs, 5 Hz), PH (rehydrated yeasts treated by high PEF strength, 10.0 kV/cm, 30 µs, 1Hz), NR (rehydrated yeasts with no PEF treatment), and NI (Inactivated yeasts with no PEF treatment)], three enzyme dosages (0, 1 and 7.5U β-glucanase) and one stirring frequencies (every 4 days). The experiment was carried out in triplicate. This experiment was conducted using the model wine. The treated model wine samples were kept in Schott bottles (bottle capacity: 1 litre). All bottles were incubated in the Contherm precision environmental chamber (54000RHS, Contherrm Scientific Ltd, New Zealand) at 20 °C for 56 days. The samples were randomly divided into three blocks to avoid the effects of cold or hot spots in the incubator.

5.3 Materials and methods

5.3.1 Preparation of model wine

A model wine solution was prepared as described by Martínez-Rodríguez and Polo (2000). The model wine solution contained ethanol (10%, v/v), tartaric acid (4 g/L), malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate (0.025 g/L). The pH was adjusted to 3.0 with sodium hydroxide. Model wine were pre-poured in 1 L Schott bottles.

5.3.2 Addition of wine lees

Details of the wine lees materials and their preparation are given in Chapter 3 (Section 3.1). All wine lees were added to model wine for the final concentration of 5% (w/v). Inactivated dry yeasts were added to model wine directly. Active dry yeasts (AWRI R2) were rehydrated according to the manufacturer's instruction, followed by a washing step described in Section 5.2.1 with DI water. The prepared rehydrated yeasts were treated in the pulsed electric field, and then suspended in model wine.

5.3.3 Enzyme addition, stirring and sampling

Three enzyme dosage rates were utilized, 0 U, 1U and 7.5 U. The enzyme solution A (for 1 U dosage rate) was prepared by dissolving 40 mg of β-glucanase (Sigma-Aldrich, USA) in 2 mL of model wine solution. The enzyme solution B (for 7.5 U dosage rate) was prepared by dissolving 300 mg of β-glucanase in 2 mL of model wine solution. For the enzyme dosage of 1 U, 50 µL of the enzyme solution A was added to model wines by pipetting; For the enzyme dosage of 7.5 U, 50 µL of the enzyme solution B was added to model wines by pipetting. For the enzyme dosage of 0 U, an addition of 50 µL of model wine solution was used instead of the enzyme solution.

Model wines were stirred using an orbital shaker (MaxQ 4000, Thermo Scientific, USA) for 10 minutes at 150 rpm in an incubator. Samplings (20 mL) were carried out after stirring on every 8th day. After stirring, 20 mL of sample was transferred to a 50 mL centrifuge tube by pipetting. After sampling, the model wine was immediately sparged with nitrogen (flowrate: 2L/min) for 15 seconds. Samples were then centrifuged at 4500 g (Dupin et al., 2000), and subsequently the supernatant was subdivided into centrifuge or Eppendorf tubes, and stored at -20 °C.

5.3.4 Chemical analysis

Materials and methods are described in Chapter 3 (Section 3.1). The strength of PI staining, the absorbance of 260 nm, 280 nm, 600 nm (OD 600), the concentrations of total protein, primary

amino acid nitrogen, free amino acids, neutral and acidic polysaccharide, mannoprotein and β-glucan, and glycerol were determined as described in Chapter 3 (Section 3.2.1).

5.3.5 Statistical analysis

Analysis of variance (ANOVA) of the repeated measurements was calculated with Genstat 16 (VSN International Ltd, UK). Statistical results for total protein concentration are used as an example to illustrate the output from this analysis (Appendix II). Principle Component Analysis (PCA) was performed using XLSTAT (2017, Microsoft, USA).

5.4 Results and discussion

5.4.1 Experiment I

Effect of washing step and temperature on lysis of yeast cells

According to Martínez et al. (2016), the optical density (OD 600) of the yeast suspension represents the level of living cells; and, the absorbance of the suspending medium at 260 nm and 280 nm corresponds with the absorbance maxima of nucleic acids and proteins, respectively. In the current study, no change in the absorbance at 600 nm, 260 nm and 280 nm were expected if no lysis of yeast cells at different temperatures occurred; otherwise, a decrease of absorbance at 600 nm and an increase of 260 nm and 280 nm were expected to demonstrate increased lysis of the yeast.

The solution used for washing the yeast cells, incubation temperature and their interaction were found to significantly affect the absorbance at 600 nm, 260 nm and 280 nm ($P < 0.001$). Figure 5.2A shows the change in OD 600 of lees yeast incubated at different temperatures. The highest OD 600 was found in the samples that had no washing step, and there were no significant difference among the samples incubated at different temperatures except for the one incubated at 25°C. This indicated that the lysis of yeast cells was limited without the washing step prior to incubation. A similar trend was also observed in both DI water and 0.9% NaCl solution washing steps, but a lower OD 600 was found in the DI water and 0.9% NaCl solution compared to no washing samples. This might be attributed to the loss of yeast cells during the washing steps.

Figure 5.2B shows the release of nucleic acids from the lysed yeast cells after incubation at different temperatures. The lowest absorbance at 260 nm was found in the samples without washing step and there were no significant difference between the samples incubated at temperatures between 10 and 30°C. This is in agreement with the results of OD 600 where limited yeast cells lysis took place. More nucleic acids were released from the samples washed with DI water and 0.9% NaCl

solution. This suggested that some yeast cells were lysed due to the change in osmotic pressure during the washing steps. In addition, the absorbance at 260 nm was generally stable at different incubation temperatures, except at 30°C where the absorbance was higher in the sample washed with 0.9% NaCl solution. A similar trend of absorbance was also observed at 280 nm (Figure 5.2C). More proteins were released into the washed samples, and the highest release of protein was found in the samples washed with 0.9% NaCl solution and incubated at 30°C.

This study demonstrated that the washing step caused loss of yeast cells. However, the washing step was necessary in the current study for the removal of impurities introduced during the production of the dry active yeasts (Quirós et al., 2012). The impurities could increase the conductivity of the suspension containing rehydrated yeasts that may lower the effectiveness of PEF treatment. DI water was the chosen solution for the washing step due to its lower conductivity compared to the 0.9% NaCl solution. This study also suggested that the temperature that might be generated during PEF treatment should be maintained below 30°C. The prepared rehydrated yeasts should be stored in bottles and placed on ice to avoid excessive heating during PEF treatment.

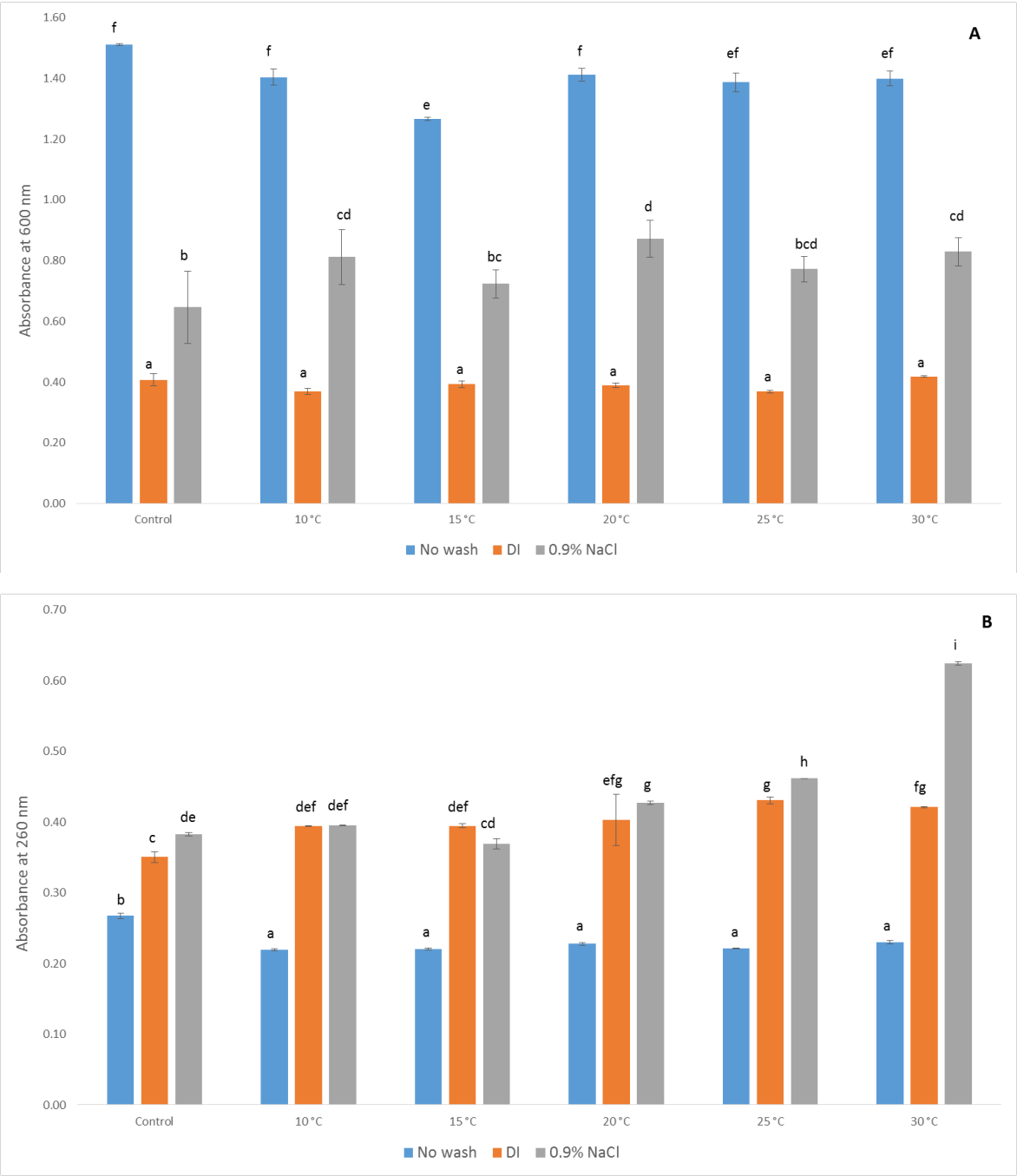


Figure 5.2 Effect of incubation temperature on absorbance at 600 nm, 260 nm and 280 nm of the rehydrated yeasts with no wash (A), washed with DI water (B) and 0.9% NaCl solution (C). The dilution factor for the measurement at 600 nm, 260 nm and 280 nm were 1000, 100 and 100, respectively. The error bar represents the standard deviation (n=3). The columns not sharing the same letters (a – i) were significantly different.

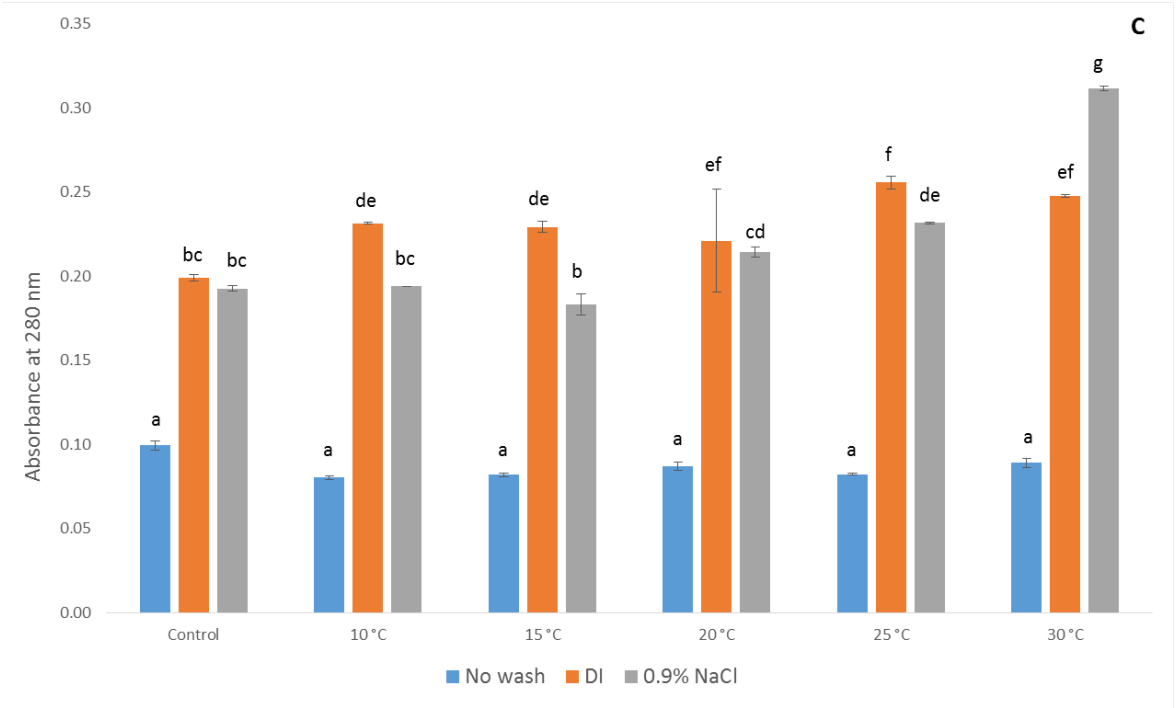


Figure 5.2 continues.

Optimization of the processing parameters of PEF treatment

Table 5.2 summaries the results of PI strength of PEF induced yeast cells and the yeast cells incubated at different temperatures (heated control samples). The Propidium Iodide (PI) strength of PEF induced yeast cells ranged from 1323 ± 31 to 1508 ± 18 which is higher than the control samples (1160 ± 5 to 1231 ± 9). This clearly demonstrated the electroporation of yeast cells by the PEF treatment. In addition, this also showed that the lysis of the yeast cells was not caused by the rising of the temperature during PEF treatment.

PEF strength significantly affected the percentage of permeabilized yeast cells ($P < 0.05$). Figure 5.3 shows the response curve of percentage of permeabilized yeast cells for different PEF strength and treatment time combinations. During PEF treatment, the percentage of permeabilized yeast cells increased with the increasing in the PEF strength. The maximum percentage of permeabilized yeast cells can be achieved by applying PEF strength (10 kV/cm). However, the percentage of permeabilized yeast cells did not increase by increasing the treatment time. This suggested that the yeast cells may be heavily damaged in the electric field, of which the cells were not able to trap the PI stains.

Table 5.2 Summary of P.I strength of the yeast cells with/without PEF treatment.

PEF strength (kV/cm)	Treatment Time (s)	Frequency (Hz)	Temperature	P. I strength
5.5	1	5		1323.00±31.24
1	15.5	5		1328.33±36.07
5.5	15.5	3		1331.33±40.50
5.5	15.5	3		1378.33±25.40
1	1	3		1324.33±32.56
5.5	30	5		1417.33±47.71
1	30	3		1346.67±36.77
5.5	15.5	3		1412.67±30.92
5.5	15.5	3	room temperature	1362.67±63.41
10	15.5	1		1445.33±42.85
5.5	30	1		1431.33± 6.03
1	15.5	1		1447.67±32.13
10	15.5	5		1452.33±21.83
10	30	3		1484.33±43.65
5.5	15.5	3		1508.00±18.33
10	1	3		1499.33±34.21
5.5	1	1		1491.00±16.09
0	0	0	10 °C	1160.00±4.58
0	0	0	15 °C	1231.33±9.02
0	0	0	20 °C	1223.00±6.00
0	0	0	25 °C	1182.33±12.42
0	0	0	30 °C	1209.67±11.15

P.I: Propidium iodide.

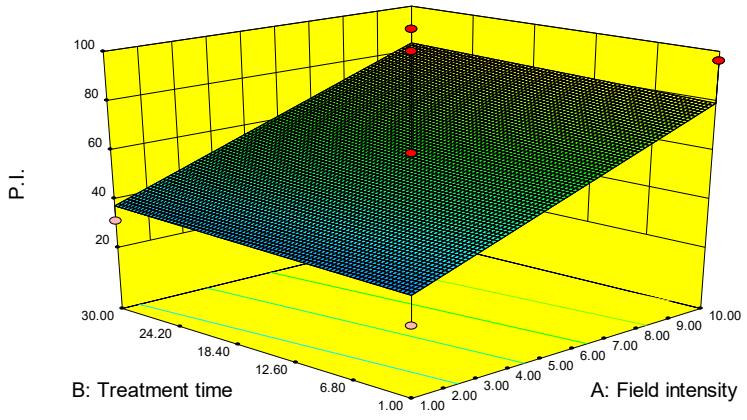


Figure 5.3 The response curve of the percentage of permeabilized yeast cells under PEF treatment with different treatment time and PEF strength. The treatment time was expressed in seconds (s); the PEF strength was expressed in kV/cm.

ANOVA was used to investigate the effects of PEF strength, frequency and treatment time on the number of living yeast cells and the leakage of UV absorbing components of the rehydrated yeasts (Table 5.3). PEF strength and frequency seems not to have had a significant impact on the number of living yeasts cells (OD600) ($P > 0.05$). Treatment time was the only parameter that had a significant impact on OD 600 ($P < 0.05$). This might be attributed to the interferences from the debris or intracellular materials released by broken yeast cells.

Table 5.3 The F-probability of ANOVA of absorbance at 600 nm, 260 nm and 280 nm.

Source of variation	600 nm	260 nm	280 nm
PEF strength	0.098	<0.001	<0.001
Frequency	0.125	<0.001	<0.001
Treatment time	0.003	<0.001	<0.001
PEF strength.Frequency	0.672	<0.001	<0.001
PEF strength.Treatment time	0.630	<0.001	<0.001
Frequency. Treatment time	0.991	<0.001	<0.001

Table 5.4 summarizes the results of the released nucleic acid and protein under different processing parameters of PEF treatment. PEF strength, frequency and treatment time and their interactions all had significant impacts on the leakage of UV absorbing components, nucleic acids (260 nm) and protein (280 nm) ($P < 0.001$). At a higher PEF strength, more yeast cells were electroporated and more nucleic acids and proteins were released. For example, the strong treatment (10 kV/cm, 30 s, 3 Hz) resulted in higher absorption units at 260 nm and 280 nm (2.121 and 0.928, respectively) compared with the mild treatment (1 kV/cm, 30 s, 3 Hz) absorbance (0.321 and 0.177).

More UV absorbing components can be released in the PEF treatment with a longer treatment time. For example, PEF treatment at 5.5 kV/cm, 1 s, and 5 Hz resulted in 0.505 and 0.256 absorption units at 260 nm and 280 nm, respectively; the absorption increased to 1.375 and 0.607 once yeast cells were treated for 30 seconds. In addition, the higher absorbance units at 260 nm that were found after a longer treatment time suggested more nucleic acids were released indicating a higher number of dead yeast cells. This confirmed the suggestion that the severely damaged yeast cells were not able to trap the P.I stains.

Table 5.4 The released nucleic acid and protein under different processing parameters of PEF treatment.

PEF strength (kV/cm)	Treatment Time (s)	Frequency (Hz)	260 nm	280 nm
5.5	1	5	0.505±0.004	0.256±0.003
1	15.5	5	0.324±0.002	0.177±0.000
5.5	15.5	3	1.251±0.001	0.556±0.000
5.5	15.5	3	1.242±0.001	0.553±0.000
1	1	3	0.325±0.003	0.177±0.002
5.5	30	5	1.375±0.000	0.607±0.001
1	30	3	0.321±0.004	0.177±0.003
5.5	15.5	3	1.225±0.000	0.547±0.000
5.5	15.5	3	1.197±0.001	0.535±0.000
10	15.5	1	2.091±0.004	0.916±0.003
5.5	30	1	1.012±0.006	0.460±0.003
1	15.5	1	0.337±0.002	0.188±0.000
10	15.5	5	2.128±0.002	0.953±0.003
10	30	3	2.121±0.003	0.928±0.003
5.5	15.5	3	1.234±0.005	0.549±0.000
10	1	3	0.754±0.005	0.361±0.004
5.5	1	1	0.335±0.001	0.184±0.001

A similar finding was also found at higher frequencies. For example, at a frequency of 5 Hz (5.5 kV/cm, 30 s), more UV absorbing components were released compared with 1 Hz. In addition, the

frequency of PEF treatment should be a minor factor compared with PEF strength and treatment. At 5 Hz, the absorption units increased from 1.012 and 0.460 to 1.375 and 0.607 at 260 nm and 280 nm, respectively.

The highest UV adsorption units at 260 nm (2.091 to 2.128) and 280 nm (0.916 to 0.953) were found in the samples treated at the highest conditions (10 kV/cm, 15.5 – 30 s, 1 – 5 Hz) in the current study. This is consistent with the results of the percentage of permeabilized cells. Therefore, such processing parameters should be used for the electroporation of the rehydrated yeast.

5.4.2 Experiment II

5.4.2.1 Total protein

The total protein concentration was determined for an aging period of 56 days. Samples were not collected after day 56 because values appeared to have plateaued. In comparison with Aging experiment I (Chapter 3), the changes in the total protein concentration of inactivated yeasts and rehydrated yeasts were found to be similar for I-4 and I-ENZ-4, and R-4 and R-ENZ-4, respectively. In addition, the lees material, enzyme treatments, and most interactions had a significant effect ($P < 0.05$) on total protein concentration in the model wine. In contrast, Aging experiment II provides new information on the effects of PEF treatment on protein released during aging on lees. Overall, PEF strength, enzyme treatments, time and their interactions all had a significant impact on total protein concentration in model wine ($P < 0.05$) (Table 5.5).

Table 5.5 The results of the ANOVA of total protein concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
PEF strength	< 0.001
Enzyme	< 0.001
PEF strength.Enzyme	< 0.001
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	< 0.001
Time.Enzyme	< 0.001
Time.PEF strength.Enzyme	0.002

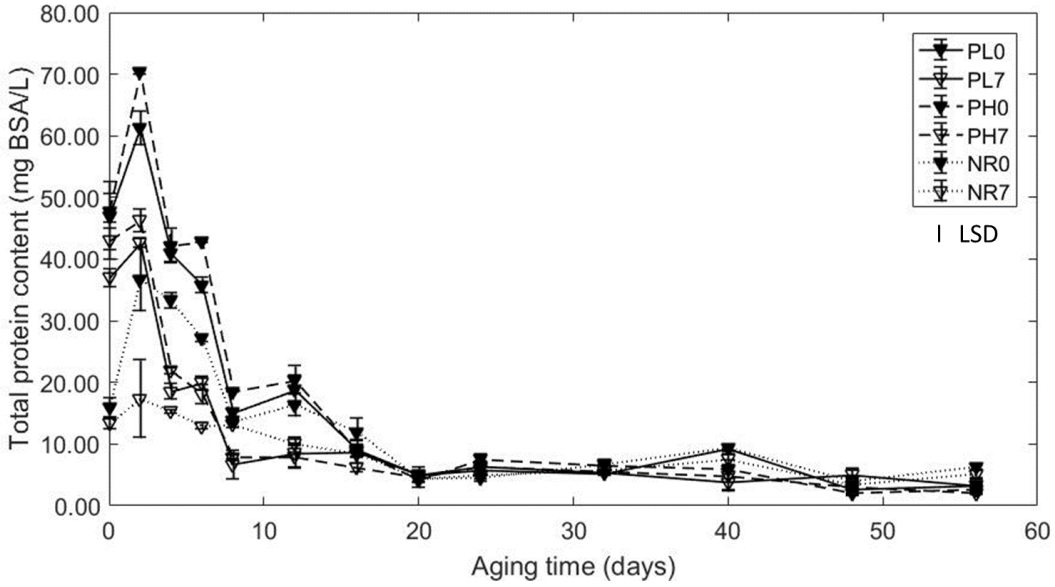


Figure 5.4 The total protein concentration of model wines containing rehydrated yeasts induced with PEF treatment and enzyme.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. Number 0 represents no enzyme addition. Number 7 represents 7.5U of enzyme addition. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

Figure 5.4 shows the changes of the total protein concentration of the model wines containing PEF treated yeasts and β-glucanase addition throughout the aging experiment. In general, an increase in protein concentration was observed in the model wine containing rehydrated yeasts during the first two days; then the protein concentration started to decrease and reached a plateau (< 10 mg BSA/L) on Day 20 of the aging experiment. During the first two days, the highest total protein concentration was found in PH0 (70.35 mg BSA/L), followed by PL0 (40.35 mg BSA/L), and PH7 (46.33 mg BSA/L) and PL7 (42.69 mg BSA/L). The lowest total protein concentration was found in NR0 and NR7, which were 36.76 and 17.43 mg BSA/L, respectively.

PEF treatment significantly affected the release of protein from rehydrated yeasts during the aging experiment ($P < 0.05$). Figure 5.4 shows that the concentration of protein increased drastically during the first two days of aging in the model wine containing PEF treated yeasts (PH0 and PL0), which were 91% and 67% higher than the model wine containing untreated yeasts (NR0). PEF strength also significantly affected the release of protein from rehydrated yeasts during the aging experiment ($P < 0.05$). The yeasts treated with PEF at high strength (10 kV/cm) released more

protein into the model wine than the ones with lower PEF strength (5.5 kV/cm). As explained in the aging experiment in Chapter 4, proteins can be passively released from yeast by exorption. There was no exorption process in the PEF treated yeasts due to the loss of cell integrity caused by electroporation, and more protein can be released through these pores. Furthermore, the application of higher PEF strength on yeasts might causes more severe damage on yeast cell membranes than lower PEF strength, thus the release of more protein. In the following days of aging, PEF treatment may also had impacts on the decrease of the total protein concentration during the early stage of aging. For example, the total protein concentration of the model wine containing the PEF treated yeasts (PH0 and PL0) decreased sharply from Day 2 to Day 4; where the protein concentration of the model wine containing the untreated yeasts (NR0) decreased at a much slower rate. Electroporation can cause plasmolysis of the organelles and the release of enzymes; this facilitated the contact of the released enzymes to target compounds or molecules (Martínez et al., 2016). For example, more proteases could be released from the damaged cells and hydrolyse the released proteins at a higher rate. However, this explanation may require further confirmation in future studies.

The interaction between PEF treatment and enzyme significantly affected the release of protein from rehydrated yeasts during the aging experiment ($P < 0.05$). The total protein concentration of the model wine containing PEF treated yeasts with the addition of enzyme (e.g. PH7 and PL7) was lower than the ones without enzyme addition (e.g. PH0 and PL0) until Day 20. The rupture of yeast cell walls by electroporation not only favoured the release of intracellular enzymes, but might also introduced more binding sites for these enzymes. The introduction of the additional β-glucanase then leads to the further damage of yeast cell walls. Therefore, the lower concentration of the total protein was observed as the consequence of more protease being released. In term of the total protein concentration, the interaction between PEF treatment and enzyme might also accelerate the yeast autolysis and aging process. For example, the total protein concentration of the model wine containing PH7 and PL7 reached a plateau on Day 8 which is about 12 days earlier than the other samples.

5.4.2.2 Free amino acids

Total free amino acids

In the current study, the sum of free amino acids of the model wine with the different treatments [lees materials (inactivated yeasts, yeasts treated with high PEF strength, yeasts treated with low PEF strength, and untreated yeasts) and enzyme addition (0U, 1U and 7.5U of β-glucanases per treatment unit)] was determined throughout the aging period of 56 days. ANOVA showed that aging

time, enzyme concentration and lees materials and their interactions significantly influenced ($P < 0.05$) total free amino acids (Table 5.6). Lees material had the biggest effect on the concentration of free amino acid. Although enzyme addition had significant impacts on the concentration of total free amino acid, the differences in mean concentrations were found to be small; and the overall mean of the concentrations of the total free amino acid in the model wine with different enzyme addition were 1245, 1255 and 1266 µmol/L, respectively.

Table 5.6 The results of the ANOVA of total free amino acids in model wines with different treatments.

Source of variation	F probability
Block.Subject stratum	
Enzyme	0.002
Lees material	< 0.001
Enzyme.Lees material	0.023
Block.Subject.Time stratum	
Time	< 0.001
Time.Enzyme	0.041
Time.Lees material	< 0.001
Time.Enzyme.Lees material	0.013

Aging time affected total free amino acids in the model wine. A decreasing trend was observed in the model wine containing inactivated yeasts (NI) (from approximately 2300 µmol/L at Day 0 to 1640 µmol/L at Day 56) (Figure 5.5). In PL, PH and NR, the total free amino acids increased from approximately 300 µmol/L at Day 0, and reached 1000 µmol/L at Day 32 and became constant until the end of aging time. The impact of aging time on total amino acids was also reported by Martínez-Rodríguez and Polo (2000), Suárez et al. (2005) and Torresi et al. (2014) where the release of free amino acids from *Saccharomyces bayanus* (EC1118) into model wine, sparkling wine and sparkling cider, respectively was investigated and it was found that aging time had a significant impact on total free amino acid concentrations. For example, Martínez-Rodríguez and Polo (2000) demonstrated that the total free amino acids was increased from 68 at Day 0 to 289 mg/L at Day 2, and became constant and finally reached 315 mg/L at Day 15.

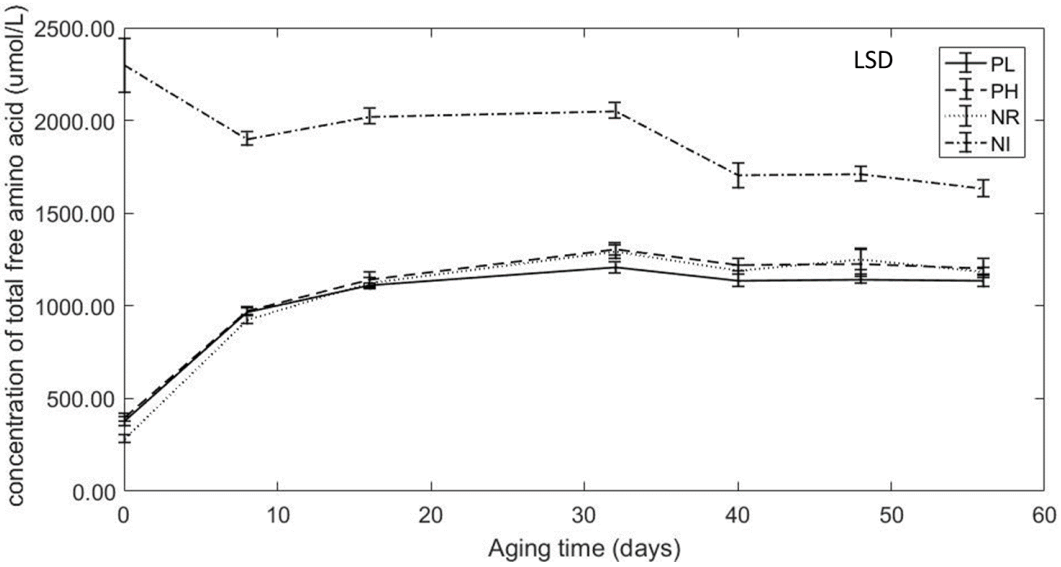


Figure 5.5 The changing of total free amino acids of the model wine containing different lees materials. NI: inactivated yeasts; PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 9). The black bar represents the least significant difference (LSD).

The differences in total free amino acids evolution trends between NI, and rehydrated yeasts (PL, PH, and NR) can be attributed to the lees type as they differ in the inactivation treatment and yeast strains. The inactivation treatment of NI enabled a quick release of amino acids to the model wine at Day 0 (2300 µmol/L). In addition, NI was manufactured using a selected yeast strain that may contain more amino acids than the rehydrated yeasts used in the current study. The total free amino acids of PL and PH and NR were very similar because they were the same yeast strain. Bozdoğan and Canhaş (2012) compared the concentrations of free amino acid from *S. cerevisiae* and *S. bayanus* during the aging of sparkling wine and confirmed that yeast strains significantly affect the total free amino acids released. For example, the total free amino acids from the wine containing S. cerevisiae (42 mg/L) had higher concentration than the one containing *S. bayanus* (32 mg/L).

Both an increase and a decrease in total free amino acids were observed. The increasing of the total free amino acids can be attributed to passive exsorption and the hydrolysis of nitrogenous compounds (Martínez-Rodríguez & Polo, 2000; Guilloux-Benatier & Chassagne, 2003). In the current study, exsorption might only occur for the model wine containing untreated yeasts (NR) within the early stage of aging. This is because of the integrity of untreated yeasts cell walls, whereas PH and PL had lost cell integrity during the inactivation process and the increase of free amino acids must be due to the hydrolysis of nitrogenous compounds. A decrease in free amino acids concentration had been explained by previous studies by the mechanisms of deamination reactions of amino acids or

participation in the formation of different compounds (Martínez-Rodríguez et al., 2002; Bozdoğan and Canbaş, 2012).

In more detail, the information of the effects of PEF treatments, enzyme and aging time on the total free amino acids concentration in the model wine was demonstrated for the first time (Table 5.8). Overall, the aging time and interaction between aging time and PEF strength had a significant impact on the free amino acid concentration in model wine ($P < 0.001$). Enzyme and its interaction with time and PEF strength had no significant impact on total free amino acids concentration ($P > 0.05$). Although there is a significant impact of PEF treatment on total amino acids, the numerical differences were small; and the overall mean concentrations of the total free amino acid in the model wine containing the rehydrated treated under different PEF strength was 1037, 1068 and 1013 µmol/L, respectively.

Table 5.7 The results of the ANOVA of total free amino acids in model wines containing PEF treated and untreated yeasts.

Source of variation	F probability
Block.Subject stratum	
PEF strength	< 0.001
Enzyme	0.684
PEF strength.Enzyme	0.271
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	< 0.001
Time.Enzyme	0.125
Time.PEF strength.Enzyme	0.055

Primary amino acid nitrogen (PAN)

Separately from total free amino acids, primary amino nitrogen (PAN) concentrations were also determined. The PEF strength, enzyme addition (β-glucanase) and their interaction were found significantly to affect PAN throughout aging experiment of 56 days ($P < 0.001$) (Table 5.8). Although PEF strength, and its interactions with time and/or enzyme addition showed a statistical significant impact on PAN, the magnitude of these effects were small compared with the other treatments.

Table 5.8 The results of the ANOVA analysis of PAN in model wines.

Source of variation	F probability
Block.Subject stratum	
PEF strength	< 0.001
Enzyme	< 0.001
PEF strength.Enzyme	< 0.001
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	< 0.001
Time.Enzyme	< 0.001
Time.PEF strength.Enzyme	< 0.001

The greatest PAN concentrations were observed in the PL, PH and NR treatment (up to 60 mg/L); and the PAN concentrations were similar between these treatments. This might be attributed to the same yeast strain was used in these treatments. Concentrations determined in treatment NI were much lower (approximate 35 mg/L). The low PAN concentrations in the NI treatment can be attributed to loss of integrity of yeast cells, e.g. nitrogen compounds of inactivated yeasts were possibly lost during the inactivation process. In comparison with aging experiment in Chapter 4, the concentrations of PAN of rehydrated and inactivated yeasts were found to be similar in R-4 and R-ENZ-4, and I-4 and I-ENZ-4, respectively. In contrast, Aging experiment II provides new information on effects of PEF treatment on PAN during aging on lees.

Figure 5.6 illustrates the correlation between the concentrations of primary amino nitrogen and total free amino acids for the different lees material treatments. As with total free amino acids, lees material is the major factor to affect PAN. There was a strong correlation of PAN with total free amino acids in both treatment PL, PH and treatment R. Thus, PAN increased along with the release of free amino acids from these lees materials. In treatment NI, PAN was not correlated to total free amino acids; PAN remained constant as the concentration of total free amino acids increased from 1600 to 2300 µmol/L.

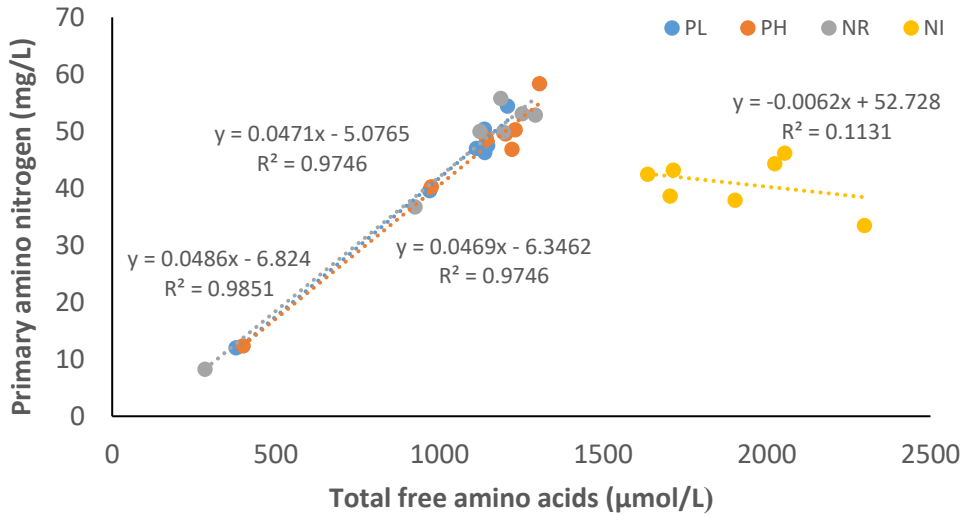


Figure 5.6 Correlation between the concentrations of primary amino nitrogen and total free amino acids in model wines containing PEF treated lees (PL and PH), Non PEF treated lees (NR), and inactivated yeasts (NI) during aging experiment.

Comparison of free amino acids profile

Principle component analysis (PCA) was performed to investigate the variability of the free amino acid composition of model wine with the different treatments [lees materials (inactivated yeasts, PEF treated yeasts, and untreated yeasts) and enzyme addition (β-glucanases)] (Figure 6.3.4). The first two principle components explained 56 % and 30% of the variance in the dataset, respectively.

According to Figure 5.7, the variation in the free amino acid composition of model wine containing different lees material can be easily distinguished. All samples from the model wine containing inactivated yeasts (NI) had a positive count on F2 and a negative count on F1. This indicated that, on average, the samples containing inactivated yeasts were different from PEF treated and untreated yeasts in terms of different types of amino acid released, which can be attributed to the differences between different yeast strains. In more detail, the samples containing inactivated yeasts had higher concentrations of Pro, Glu, Gln, Ala, Arg and His. The samples containing PEF treated and untreated yeasts had a higher concentration of Thr, Gly, Tyr, Met, Phe and Leu.

The variation in the free amino acid composition at different times of aging can be distinguished. For the model wine containing inactivated yeasts, samples from Day 0 was separated from the ones collected in the other days. However, the samples from Day 8, 16, 32, 40, 48 and 56 were poorly separated. This might indicate that, on average, the variation in amino acids composition was limited throughout the most of the aging experiment. For PEF treated and untreated yeasts, samples from Day 0 and Day 8 had a negative count on both F1 and F2, which were well separated from the

samples from Day 16 to Day 56 (all had a positive count on F1). Two interpretations can be made based on observation. First, the concentration of the different free amino acids in the initial days (Day 0 and 8) was lower than the average; and Ala, Glu, Gln, Pro and Cys were the major amino acids. Second, as aging time increased, the composition of free amino acid changed, and Thr, Gly, Met, Leu and Lys became the major amino acids until the end of the aging experiment.

PEF treatments seem to have their effects on free amino acids composition during the early stage of the aging experiment. For example, for Day 0, the untreated lees were separated from PEF treated ones. However, the PEF treatments seem to have no effects on free amino acids composition in the later stage of the aging experiment as indicated by the poor separation between PEF treated and untreated samples. This might also suggest that the difference between amino acids composition of PEF treated and untreated yeasts became smaller as consequence of the release of proteases from untreated yeasts.

In general, the PCA results suggested that free amino acids composition in the model wine can be affected by lees material and aging time; and PEF treatment might have effects on free amino acids compositions at the initial days of aging. To investigate the variation of free amino acids during aging, the variation of concentration of total free amino acids, individual amino acids among different treatments are discussed in the later sections. Moreno-Arribas et al. (1998) expressed the results of free amino acids in molar distribution. This was done to avoid differences in the free amino acid concentrations in four wine varieties and facilitated the comparison. Table 5.9 reports the molar percentage of each amino acid at different times of aging for each lees material. In general, the molar percentage of amino acids in the model wine containing PH, PL and NR were similar to each other but different from the one containing NI. This might suggest that PEF treatment had no impact on the distribution of free amino acids during yeast autolysis.

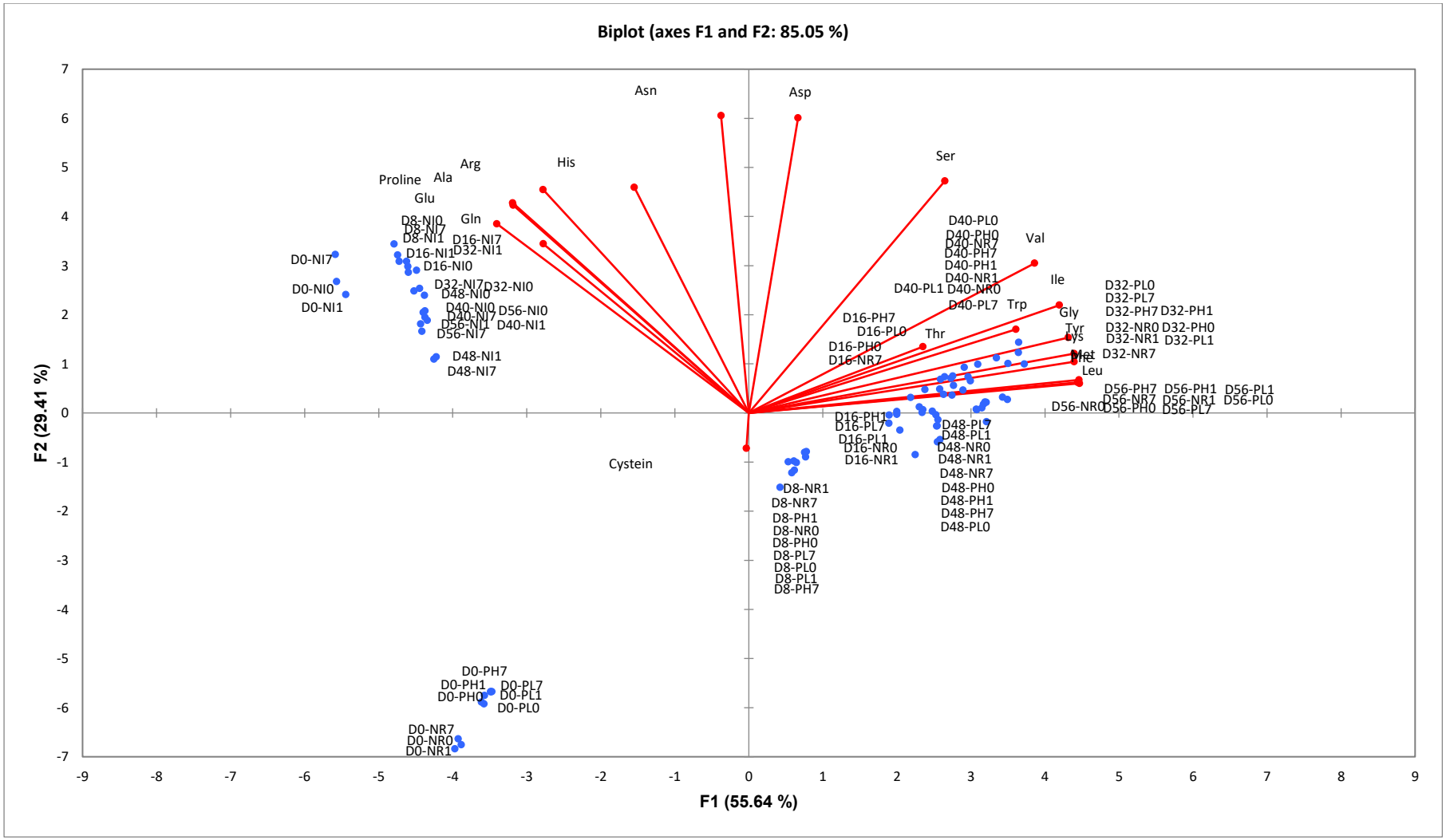


Figure 5.7 Principle component analysis (PCA) biplot of the effect of lees treatments on the free amino acids profile of the model wines. F1 represents a different combination of lees treatments; F2 represents different types of amino acids.

Table 5.9 Amino acids distribution (expressed as a molar percentage) in the model wine containing different lees material at Day 0 and Day 56 in aging.

Amino acids	NI		PL		PH		NR	
	Day 0	Day 56	Day 0	Day 56	Day 0	Day 56	Day 0	Day 56
Ala	25.32	25.11	17.70	11.34	17.38	11.09	19.34	11.48
Glu	43.10	37.76	12.64	7.87	12.99	8.68	11.77	6.45
Leu	1.20	1.48	7.05	14.82	6.92	14.73	6.60	14.91
Ser	1.76	2.15	5.81	4.04	5.68	4.06	5.40	3.67
Thr	1.33	3.24	3.43	8.81	3.51	8.39	3.76	10.02
Pro	6.37	7.38	3.08	2.61	3.00	2.63	2.97	2.59
Asp	3.28	3.41	2.10	4.81	2.36	4.77	1.72	4.89
Cys	1.50	0.03	12.28	0.21	12.10	0.18	12.43	0.22
Asn	2.31	3.29	3.65	3.57	3.63	3.55	3.77	3.52
Gln	3.77	1.27	2.81	0.64	2.60	0.63	2.52	0.58
His	0.45	0.66	1.93	0.67	1.88	0.67	2.04	0.62
Gly	1.13	1.36	4.90	3.89	4.94	3.89	5.56	4.01
Arg	3.10	4.90	3.95	2.70	4.16	2.73	4.28	2.91
Tyr	0.50	0.72	1.94	3.97	2.02	3.97	1.87	4.07
Val	1.94	2.98	4.38	6.95	4.38	6.88	3.99	6.99
Met	0.03	0.31	0.87	2.65	0.86	2.63	1.11	2.66
Trp	0.11	0.09	0.01	0.79	0.05	0.77	0.00	0.77
Phe	0.70	1.05	4.41	9.61	4.29	9.65	4.30	9.69
Ile	1.25	1.70	2.37	5.90	2.40	5.90	1.77	5.93
Lys	0.83	1.10	4.68	4.17	4.85	4.20	4.80	4.03

NI: Inactivated yeasts; PL: PEF treated rehydrated yeasts with a field strength of 5.5 kV/cm; PH: PEF treated rehydrated yeasts with a field strength of 10.0 kV/cm; NR: rehydrated yeasts with no PEF treatment.

At Day 0, Ala and Glu were the most prevalent free amino acids released in all treatments, Ala + Glu counted 68% and 30% for inactivated yeasts (NI) and rehydrated yeasts (PH, PL and NR), respectively. Leu (7%) and Cys (12%) were also prevalent free amino acids released from rehydrated yeasts. Perrot et al. (2002) studied the release of nitrogenous compounds from rehydrated yeasts (S. cerevisiae MC001 and MC002 strains) and found that Glu, α-Ala, Asp and GABA (γ-aminobutyric acid)

were the most prevalent free amino acids.

In the course of autolysis, the distribution of free amino acids released from NI was fairly similar to the Day 0. The most significant changes of the distribution of free amino acids were found in rehydrated yeasts. For example, Leu dramatically increased in the model wine containing PH, PL and NR, and became one of the most prevalent free amino acids on Day 56. This is consistent with the results reported by Perrot et al. (2002) in which Leu became the most abundant amino acids at 188 hours of autolysis. Ala and Glu decreased during autolysis but were still the most abundant amino acids at Day 56. Cys significantly decreased from about 12% to lees than 1%.

Based on the results, the distribution of free amino acids during yeast autolysis seems to be influenced by the aging time and type of yeast strains used. This is discussed in Chapter 7.

Change of free amino acids concentration during PEF induced autolysis

PEF strength had significant impact ($P < 0.05$) on most of the amino acids except Ala, Leu, Phe, Pro and Trp (Table 5.10). The change in the concentration of selected amino acids in the model wine containing PEF treated and untreated yeasts are discussed below and contribute new information on the process effects.

Table 5.10 Summary of F-probability of ANOVA of free amino acids in the model wine of aging experiment.

Amino acids	PEF strength	Enzyme	PEF strength Enzyme	Aging Time	Aging Time PEF strength
Ala	0.168	0.001	0.213	<0.001	<0.001
Arg	<0.001	<0.001	0.001	0.001	0.001
Asn	<0.001	0.184	0.144	<0.001	<0.001
Asp	0.002	0.143	0.002	<0.001	<0.001
Cys	<0.001	0.634	0.589	<0.001	<0.001
Gln	<0.001	0.342	0.726	<0.001	<0.001
Glu	<0.001	0.002	0.003	<0.001	<0.001
Gly	0.002	0.001	0.491	<0.001	<0.001
His	<0.001	0.004	0.068	<0.001	<0.001
Ile	0.015	0.081	0.102	<0.001	<0.001
Leu	0.652	0.745	0.065	<0.001	<0.001
Lys	<0.001	0.277	0.122	<0.001	<0.001
Met	0.013	0.864	0.196	<0.001	<0.001
Phe	0.267	0.244	0.077	<0.001	<0.001
Pro	0.336	0.013	0.480	<0.001	<0.001
Ser	<0.001	<0.001	0.654	<0.001	<0.001
Thr	<0.001	<0.001	0.033	<0.001	<0.001
Trp	0.036	<0.001	0.098	<0.001	<0.001
Tyr	<0.001	0.027	0.104	<0.001	<0.001
Val	<0.001	<0.001	0.060	<0.001	<0.001

Figure 5.8 shows the changing of the concentration of the selected amino acids in the model wine containing PEF treated and untreated rehydrated yeasts, including Ser, Thr, Ala, Leu, Cys and Glu. In general, although there is a significant impact of the enzyme on most of these amino acids, these differences are small compared to the overall mean values.

Both Ser and Thr were increased in all model wine during aging (Figure 5.8 A and B). the increase in Ser and Thr concentrations in the model wine confirmed that mannoprotein was degraded (Van Der Vaart et al., 1995). This observation had been reported during yeast autolysis in model wine (Martínez-Rodríguez & Polo, 2000, 2001; Perrot et al., 2002; Guilloux-Benatier & Chassagne, 2003), sparkling wine (Martínez-Rodríguez et al., 2002; Bozdoğan & Canbaş, 2012) and sparkling cider (Valles et al., 2005). Ser was found to be significantly higher in model wine containing PEF treated yeasts (about 50 µmol/L at Day 56) than untreated ones (43 µmol/L at Day 56) throughout aging. Opposite results were observed in Thr, where its concentration in the model wine containing untreated yeasts was lower than PEF treated ones. This might suggest that Thr may be more reactive than Ser and participated in the formation of new compounds. However, there is a lack of scientific research to support this assumption.

The trend of increase was observed in Ala and Leu (Figure 5.8, C and D). Although PEF treatment had significant impacts on these two amino acids, the differences were found too small compared to the overall concentrations. In comparison with the model wine containing inactivated yeasts, Ala released from inactivated yeasts was higher than PEF treated yeasts; opposite results were found in Leu in the model wines. This suggested that PEF treatment is not able to improve the concentration of the Ala and Leu; inactivated yeast is a better source for the release of Ala.

Cys was one of prevalent amino acid found in the model wine containing PEF treated and untreated yeasts at the early stage of aging (Figure 5.8 E). Although PEF treatment had a statistical impact on the changes in Cys concentration during aging, the difference was small compared to the overall concentration. The concentration of Cys during the first 48 days of aging was stable. A sharp increase started after Day 48 toward to the end of the aging experiment. Cys was a minor amino acid found in the model wine containing inactivated yeasts (approximately 35 µmol/L at Day 0), and it had a lower concentration compared to the PEF treated and untreated yeasts (approximately 48 µmol/L at Day 0); Cys was also found disappeared at the end of aging. Identification and quantification of Cys were commonly not reported in previous studies in model wine and sparkling wine, which might be due to its low concentrations towards to the end of aging. Guilloux-Benatier and Chassagne (2003) reported free amino acids released from rehydrated yeasts (Saccharomyces cerevisiae, Levuline BRG) under different autolysis condition (30 °C for 2 weeks or 18 °C for 8 weeks without stirring) in the model

wine; Cys had a 0.2-0.3 molar distribution in different autolysate.

Glutamic acid had been found related to the umami tastes in wine which also gives a sense of fullness and roundedness in the mouth; it is also being described as 'balancing and blending', giving 'continuity", thickness and a feeling of satisfaction (Klosse, 2012). Glutamic acid was a prevalent amino acid in the model wine containing PEF treated and untreated yeasts. PEF treatment had a significant impact on the release of glutamic acid. For example, the glutamic acid concentration in the model wine containing PEF treated yeasts was higher than untreated ones. However, PEF treatment was not able to increase the glutamic acid concentration in the model containing rehydrated yeast to that enrichment with inactivated yeasts (approximately 100 and 660 µmol/L at Day 56, respectively). Therefore, both PEF treatment and application of inactivated yeasts can be used to improve umami tastes in wine, with inactivated yeasts seeming to give better results.

To conclude, PEF treatment was not able to improve the concentration of total free amino acids in the model wine at the end of the aging experiment. Aging time and yeast strain were two factors that had significant impacts on total free amino acids. However, PEF treatment improved the release of glutamic acids from rehydrated yeasts to model wine.

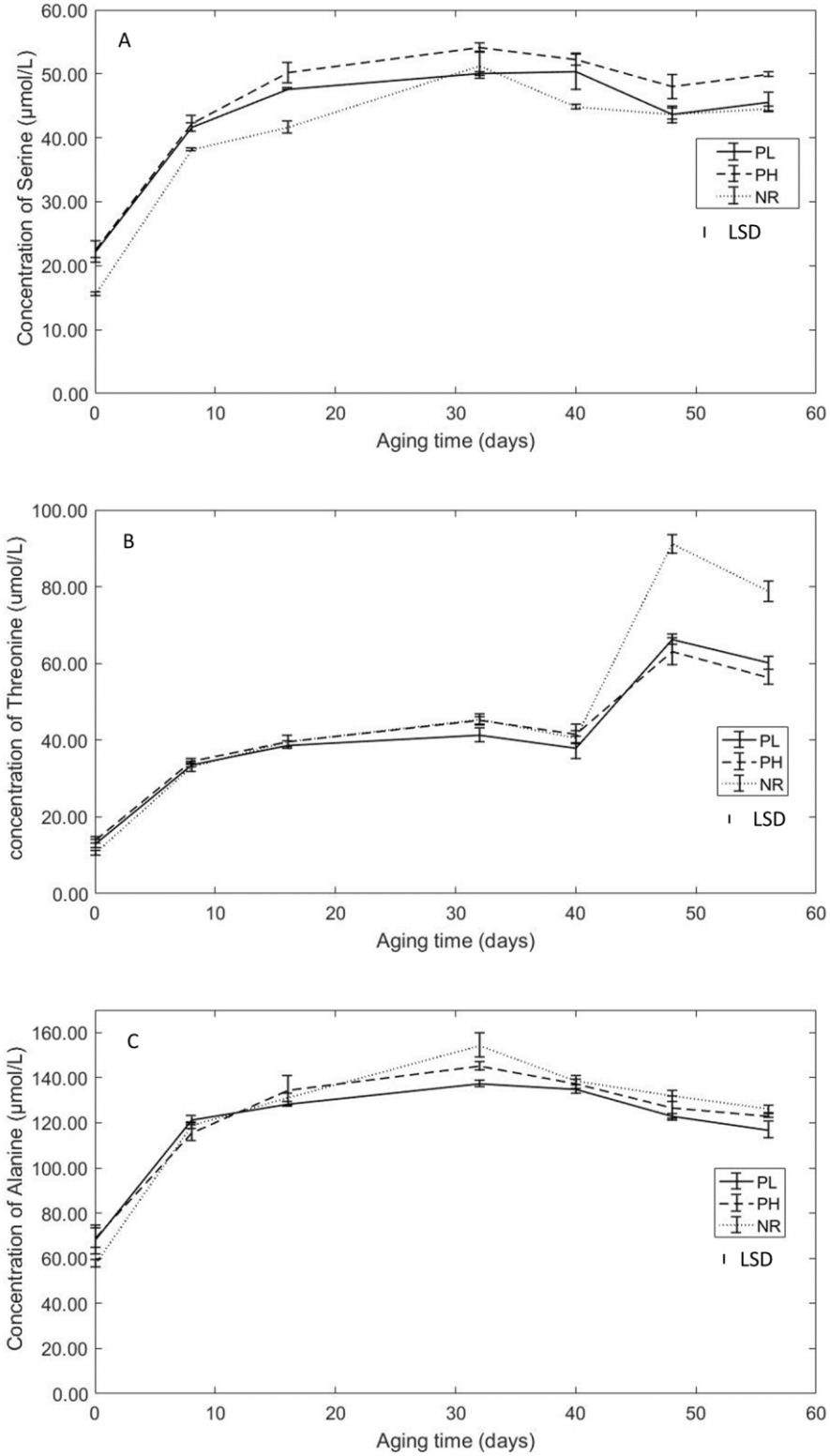
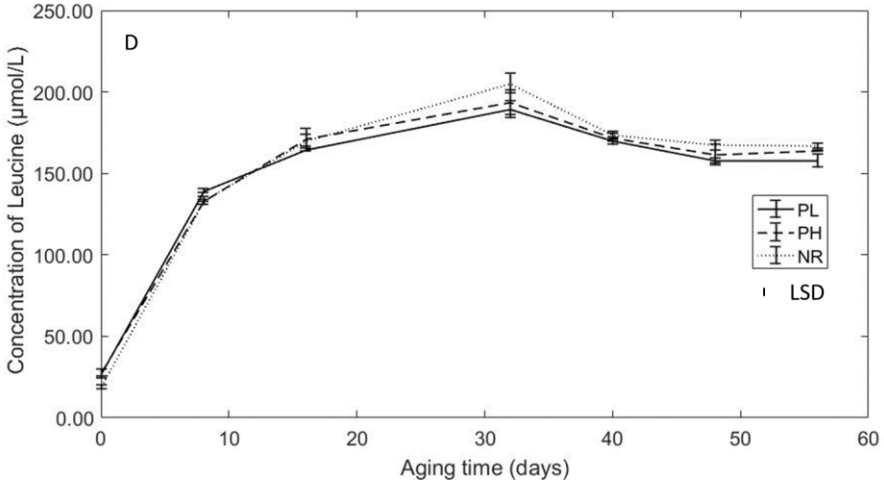
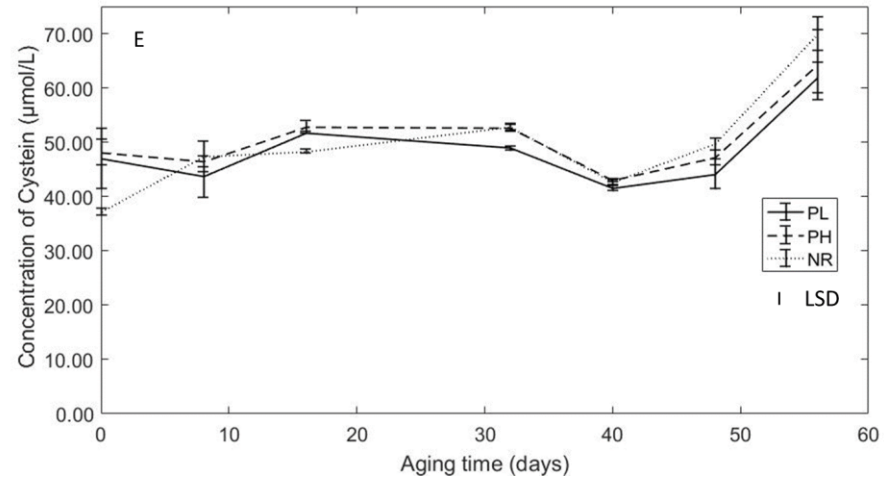


Figure 5.8 Total free amino acids of the model wine containing PEF treated and untreated rehydrated yeasts.A: Serine; B: Threonine; C: Alanine; D: Leucine; E: Cysteine; F: Glutamic acid.PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).





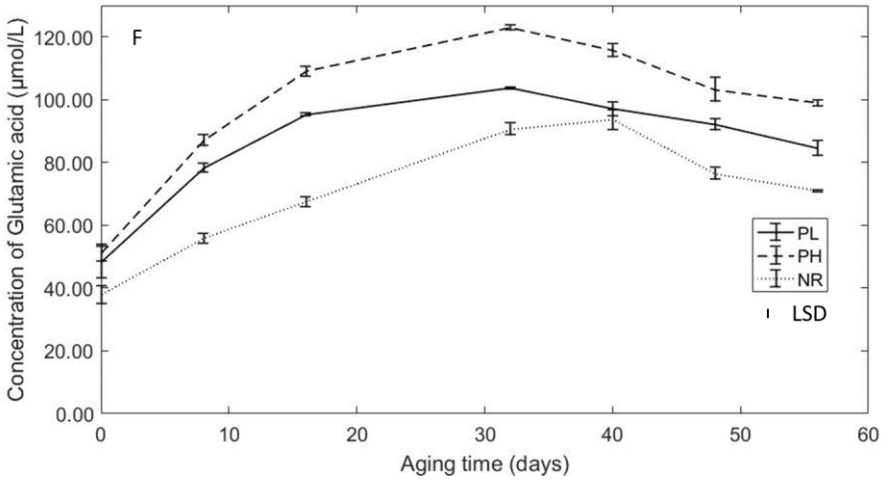


Figure 5.8 continued. Total free amino acids of the model wine containing PEF treated and untreated rehydrated yeasts.

A: Serine; B: Threonine; C: Alanine; D: Leucine; E: Cysteine; F: Glutamic acid.PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

5.4.2.3 Polysaccharides

Neutral polysaccharides

The neutral polysaccharide concentration of the model wine was determined throughout the aging period of 56 days. Analysis of variance was used to determine the variation in polysaccharide concentration due to the lees material, enzyme, and time. Overall, lees material, enzyme addition (β-glucanase), aging time and their interactions all had significant effects on the neutral polysaccharide concentration in model wine ($P < 0.05$) (Table 5.11).

Table 5.11 The results of the ANOVA analysis of neutral polysaccharide concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Lees material	< 0.001
Enzyme	< 0.001
Lees material.Enzyme	< 0.001
Block.Subject.Time stratum	
Time	< 0.001
Time.Lees material	< 0.001
Time.Enzyme	< 0.001
Time.Lees material.Enzyme	< 0.001

Figures 5.9 and 5.10 show the changes in the neutral polysaccharide concentration of model wine containing lees material. Neutral polysaccharide is the major polysaccharide found in the model wine and it was used to reveal the trend of total polysaccharide in this aging experiment. The highest neutral polysaccharide concentration was observed in the model wine containing inactivated dry yeasts (NI), followed by the samples treated by PEF treatment (PH and PL), and rehydrated yeasts (NR). An increase in neutral polysaccharide concentration was observed in NI during the first eight days; then the trend of neutral polysaccharide concentration became stable except for the highest enzyme dosage (NI7) treatment. A decrease of neutral polysaccharide concentration was observed in PH, PL and NR during the first eight days of aging, followed by a more stable trend throughout the rest of the aging period. Both an increase and a decrease of neutral polysaccharide concentration are expected during yeast autolysis. The increase of the neutral polysaccharide concentration in model wines can be attributed to the accumulation of the released polysaccharide; where the decrease of the neutral polysaccharide can be attributed to the lowering of its solubility as consequence of changing its composition during the enzymatic reaction (Pati et al., 2010).

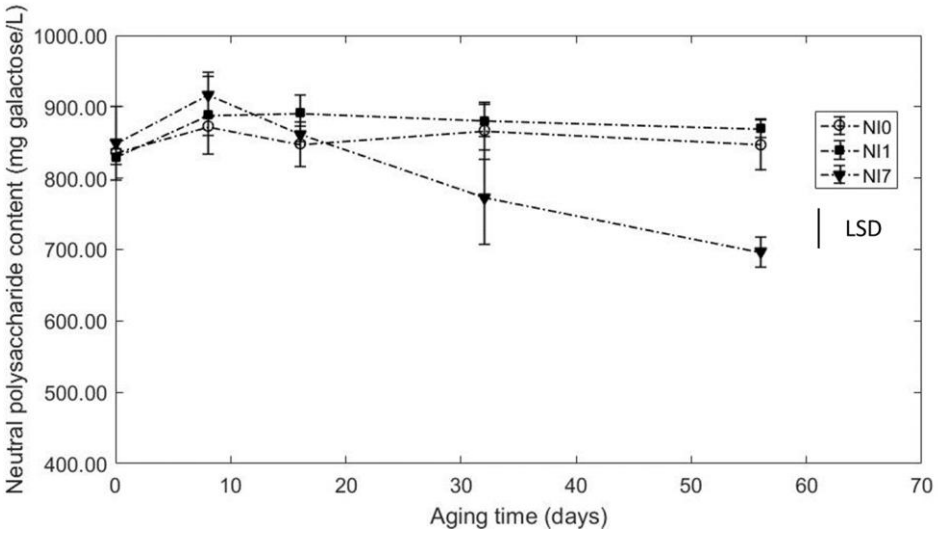


Figure 5.9 Neutral polysaccharide concentration of the model wine containing the inactivated yeasts in aging experiment.

0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

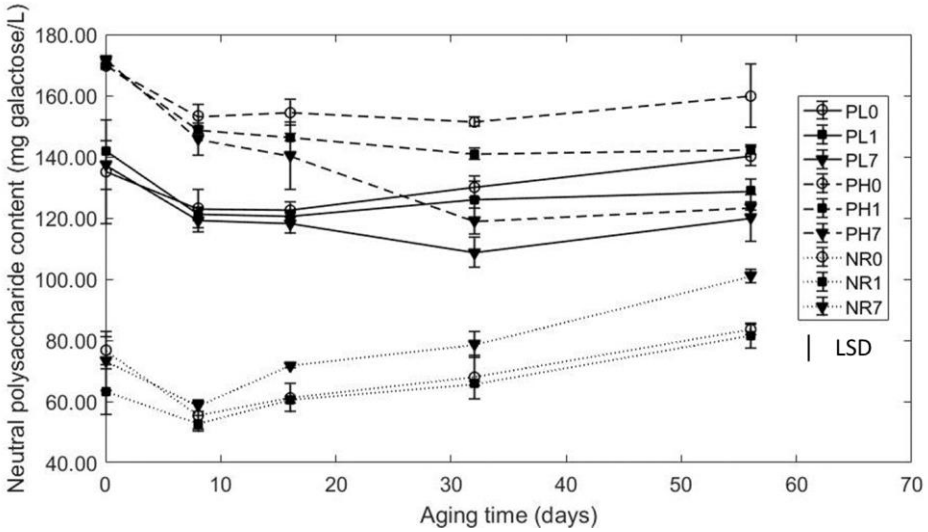


Figure 5.10 Neutral polysaccharide concentration of the model wine containing the PEF induced and non-induced yeasts and in aging experiment.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. 0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

In the current study, PEF strength significantly affected the release of polysaccharides from lees material ($P < 0.05$). For example, the highest neutral polysaccharide concentration at Day 0 was observed in the model wine containing the lees treated by high PEF strength (PH) (169.73 to 171.86

mg galactose/L), followed by low (PL) (135.15 to 141.81) and none PEF strength (NR) (63.25 to 76.65 mg galactose/L). High PEF strength (PH) improved the release of neutral polysaccharide 1.3 to 2.2 fold compare to PL and NR, respectively. This suggested that a higher strength of PEF treatment is able to cause a higher level of electroporation which leads to a greater release of polysaccharides from lees material.

The addition of enzyme significantly affected the release of polysaccharides from different lees materials type throughout the aging experiment ($P < 0.05$) in the current study. This is consistent with the finding in aging experiment in Chapter 4, where the addition of 7.5U of β-glucanase significantly decrease and increase the concentration of neutral polysaccharide in model wine containing inactivated dry yeast and rehydrated yeast throughout aging experiment, respectively. However, the effect of the addition of 1U of β-glucanase on the concentration of neutral polysaccharide was found not significant ($P > 0.05$) throughout the aging experiment. For example, the concentration of neutral polysaccharide of NI1 and NR1 were very similar to their control (NI0 and NR0) during aging.

The trends of changes in the concentration of neutral polysaccharide in the presence of β-glucanase found in the model wine with PEF induced lees (PL and PH) were different from NI and NR (Figure 5.10). Among PH and PL, the highest concentration of neutral polysaccharide was found in the model wine without additional enzyme, followed by the addition of 1U, and 7.5U of β-glucanase. In the model wine with 1U enzyme addition, the concentration of neutral polysaccharide PH1 continuously decreased from day 8 throughout the aging experiment, whereas the concentration of neutral polysaccharide was increased in PL1. In comparison, the concentration of neutral polysaccharide in PH7 and PL7 treatments continued to decrease from Day 8 to 32, followed by an increase in the next three weeks; the decrease of neutral polysaccharide concentration of PH7 was steeper than PL7.

The above observations can be attributed to the following reasons. First, the trend of neutral polysaccharide concentration during yeast autolysis is based on the availability of β-glucanase. Polysaccharides can be hydrolysed by adding more β-glucanase during yeast autolysis which resultes in a lower concentration of polysaccharide. Second, electroporation released more β-glucanase originally exists in yeast cells. This explains the significant difference in neutral polysaccharide concentration between PH1 and PH0, and between PL1 and PL0 and between NR1 and NR0. Third, higher PEF strength released more β-glucanase due to more severe damage to the yeast cells. Therefore, the sharp decrease of the neutral polysaccharide concentration in PH7 can be attributed to the rate of polysaccharide released was lower than the hydrolysis of the released polysaccharide due to its higher availability of β-glucanase.

Acidic polysaccharides

The acidic polysaccharide concentration of the model wine was determined throughout the aging period of 56 days. Analysis of variance was used to study the differences between polysaccharide concentration due to the lees material, enzyme, and time factors. There were no significant effects of the lees material, enzyme addition (β-glucanase) and aging time, and their interactions ($P > 0.05$).

Figure 5.11 shows the changes in acidic polysaccharide concentration of model wine containing different lees type throughout the aging experiment. In general, the acidic polysaccharide concentrations of all treatments were low (< 10 mg galacturonic acid/L) throughout the aging experiment; and their concentration was more or less stable in all model wine, although slight differences were found among the treatments. This is consistent with the finding in aging experiment in Chapter 4 and previous studies (Guadalupe et al., 2007; Del Barrio-Galán et al., 2011; Del Barrio-Galán et al., 2012).

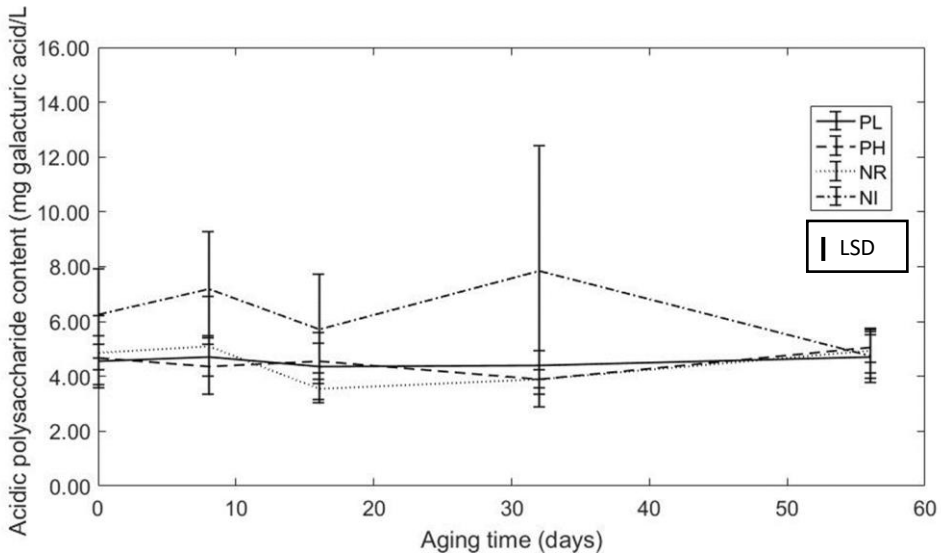


Figure 5.11 The example of acidic polysaccharide concentration of different lees materials in aging experiment.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment; NI: inactivated yeasts 0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 9). The black bar represents the least significant difference (LSD).

5.4.2.4 Mannoprotein and β-glucan

HPLC-ELSD was used to identify and quantify mannose released in the model wine containing different lees material (Figure 5.12). Mannose was successfully separated from the other sugar monomers that can be found in wine such as fructose, glucose, galactose and trehalose. However, it was not possible to be separated galactose from glucose due to its similar chemical structure and molar mass. Therefore, the galactose was quantified as glucose in the current study. The concentration of mannose and glucose were used to represent the concentration of mannoprotein and β-glucan according to the method described by Quirós et al. (2011).

Mannose and glucose were the only two sugar monomers identified in all model wines (Figure 5.13). This indicates that the collected macromolecules released from inactivated and rehydrated yeasts were composed of mannoprotein and β-glucan as a result of breaking down of yeast cell walls. This is consistent with the findings in red and white wine with on lees aging (Rodrígues et al., 2012; Quirós et al., 2012). A small amount of arabinose and rhamnose were also found in real wine sample. For example, 3.5% and 2.2% of the polysaccharides fraction (with a molecular weight of 160 kDa) were arabinose and rhamnose, respectively in white wine aged in wood barrel (Rodrígues et al., 2012). Although no data were available for illustration, the existence of arabinose and rhamnose might be from to the breakdown of the polysaccharide residues after wine fractionation process.

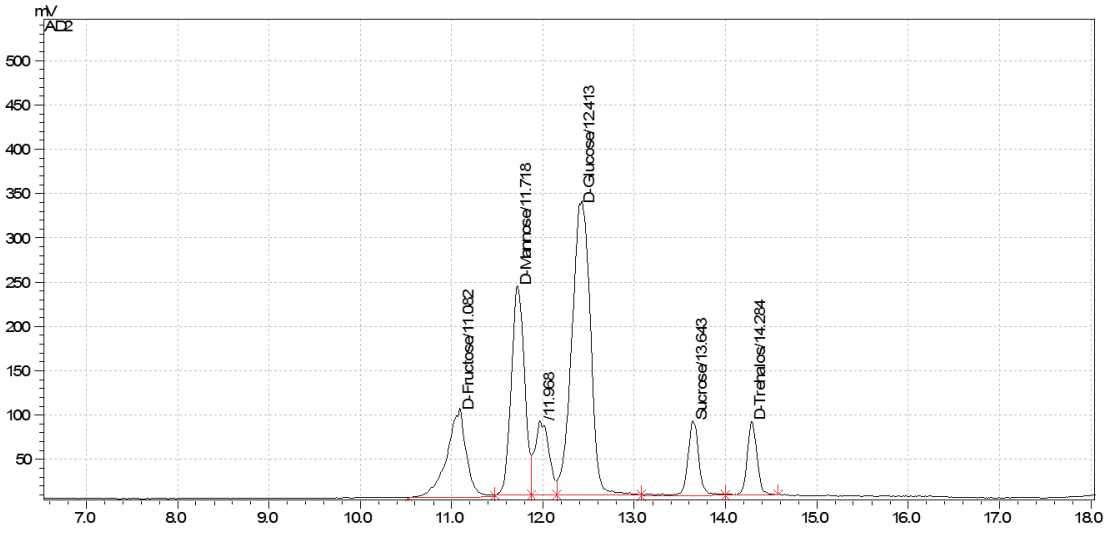


Figure 5.12 The example of the chromatogram of a sugar mix.

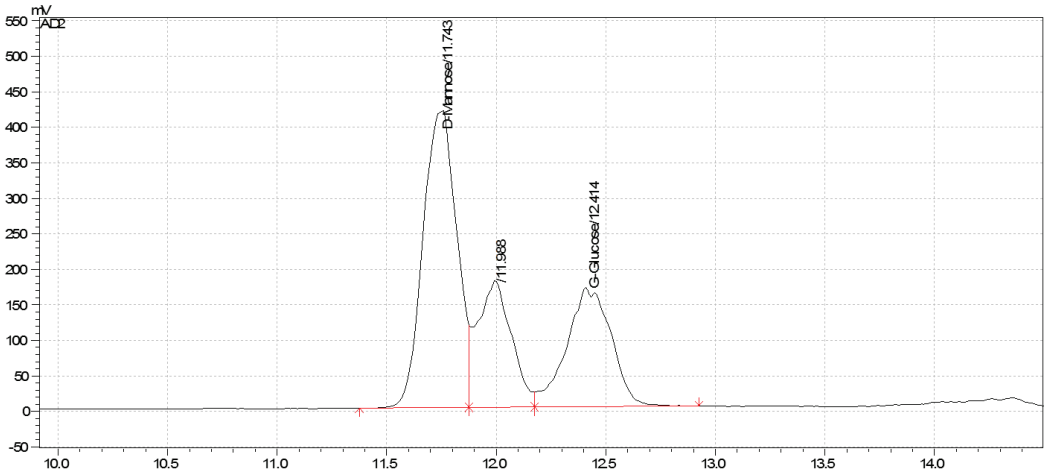


Figure 5.13 Example of identified sugars in the model wine containing inactivated yeasts, PEF and enzyme induced yeasts.

The ratio of mannose/glucose of most samples increased during aging (Table 5.12). This is consistent with a previous study on aged sparkling wine where an increase of mannose/glucose ratio was found over 18 to 30 months of aging (Martínez-Lapuente et al., 2013). The increase in the mannose/glucose ratio during yeast autolysis might suggest that glucan is able to form more unstable compounds susceptible to precipitation than mannoproteins (Martínez-Lapuente et al., 2013).

Table 5.12 The mannose/glucose ratio in the model wine containing different lees material during the aging experiment.

Sample	Day 0	Day 32	Day 56
PL0	0.00	2.61	2.98
PL7	0.00	3.35	4.32
PH0	0.00	4.80	3.47
PH7	0.00	3.74	6.15
NR0	0.00	3.28	4.84
NR7	0.00	4.51	6.57
NI0	3.67	4.42	5.07
NI7	3.68	5.39	7.36

Mannoprotein

Analysis of variance was used to study the differences between polysaccharide concentration due to

the lees material, enzyme addition (β-glucanase), and aging time. Overall, lees material, enzyme, time and their interactions all had a significant impact on mannoprotein concentration in model wine ($P < 0.05$) (Table 5.13).

Table 5.13 The results of the ANOVA analysis of mannoprotein concentration in model wines.

Source of variation	F probability
Block.Subject stratum	
Lees material	< 0.001
Enzyme	< 0.001
Lees material.Enzyme	< 0.001
Block.Subject.Time stratum	
Time	< 0.001
Time.Lees material	< 0.001
Time.Enzyme	< 0.001
Time.Lees material.Enzyme	< 0.001

Figure 5.14 demonstrated the changes in mannoprotein concentration in the model wines containing different lees material throughout the aging experiment. The highest mannoprotein concentration was found in the model wines containing inactivated yeasts (NI). The mannoprotein concentration of NI0 and NI7 increased from about 50 mg/L on Day 0 to 90 and 140 mg/L at the end of aging, respectively. A similar trend was found in the model wine containing rehydrated yeasts; the mannoprotein concentration of NR0 and NR7 increased from about 0 mg/L to 18 to 27 mg/L, respectively. In comparison with aging experiment in Chapter 4, the change of mannoprotein concentration in model wine containing inactivated (R-4 and R-ENZ-4) and rehydrated yeasts were similar to NI and NR in the current study. This supported the previous findings that lees materials and enzyme addition significantly affect the mannoprotein concentration in the model wines after aging.

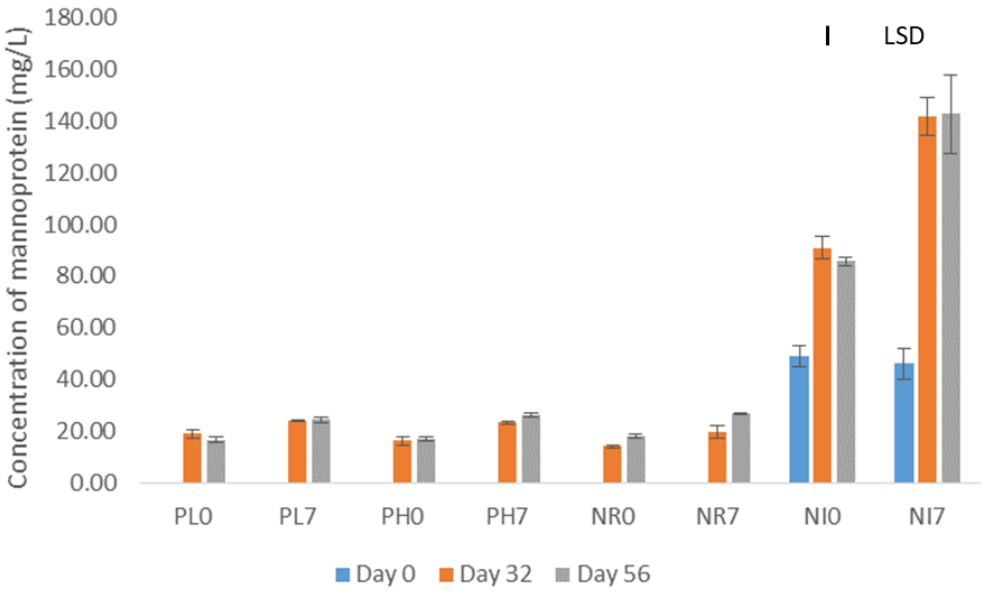


Figure 5.14 Release of mannoprotein from different lees materials during aging.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment; NI: inactivated yeasts. 0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

Table 5.14 The results of the ANOVA analysis of mannoprotein concentration in model wines containing PEF and enzyme induced yeasts.

Source of variation	F probability
Block.Subject stratum	
PEF strength	0.020
Enzyme	< 0.001
PEF strength.Enzyme	0.134
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	< 0.001
Time.Enzyme	< 0.001
Time.PEF strength.Enzyme	0.745

It is interesting to understand how PEF treatment affects mannoprotein concentration during yeast autolysis. Analysis of variance demonstrated that PEF strength or β-glucanase and interactions with aging time had a significant impact on mannoprotein concentration in model wine ($P < 0.05$) (Table 5.14). However, the interaction between PEF treatment, β-glucanase and aging time had no significant impact on mannoprotein concentration ($P > 0.05$).

In general, the mannoprotein concentration of the model wine containing PEF induced yeasts (PL and PH) increased during aging; the increase of mannoprotein concentration then slowed down or

stopped towards to the end of aging. On Day 32, the mannoprotein concentrations of the model wine containing PEF induced yeasts (PH0 and PL0) were higher than the rehydrated yeasts without PEF induction (NR0). However, there was no significant difference between the mannoprotein concentration of PH0 and PL0. In addition, the mannoprotein concentration s of the model wine containing PEF induced yeasts with enzyme addition (PH7 and PL7) were significantly higher than the PEF treated ones without enzyme addition (PH0 and PL0); and the mannoprotein concentration of PL7 was significantly higher than PH0. Such observation of mannoprotein concentration on Day 32 might indicate: First, PEF treatment on rehydrated yeasts can accelerate yeast autolysis in the term of release of mannoproteins; Second, application of 7.5U of β-glucanase with PEF treatment significantly improved the release of mannoproteins from the yeast cell walls; Third, low PEF strength can achieve similar effects on release of mannoprotein as high strength.

Furthermore, the mannoprotein concentration of PHs and PLs became constant from Day 32 to 56; whereas the mannoprotein concentration of NRs continuously increased within this period. Enzyme addition (7.5U of β-glucanase) appeared to be the main factor affecting the final concentration of mannoprotein. For example, the samples with enzyme addition (NR7, PH7 and PL7, approximately 25 mg/L) were found to be higher than the ones without enzyme addition (NR0, PH0 and PL0, 16 mg/L). The mannoprotein concentration of the samples with the same enzyme dosage were also found to be similar. This supported that the acceleration effects of PEF treatments on yeast autolysis as the sample without PEF treatment took a longer time to achieve similar level of mannoprotein concentration.

It is necessary to point out that the mannoprotein concentration of the model wine during aging was actually the balance between the amount of mannoprotein released and hydrolysed (the released mannoprotein from cell walls). In the current study, the polymeric mannose (mannoprotein) was separated from sugar monomers when passed through the size exclusion columns. The monomeric mannose was not determined in the current study. In fact, the monomeric mannose can be used to represent the hydrolysed mannoprotein during yeast autolysis as it is the only source of mannose in the model wines (mannoprotein can also be released during fermentation). Therefore, the total mannoprotein released during yeast autolysis can be estimated by determining the sum of the concentration of polymeric and monomeric mannose. This can be useful for comparing the amount of mannoprotein released by PEF treatment and conventional aging methods.

Martínez et al. (2016) investigated the influence of PEF treatments of different strength (5-25 kV/cm for 30-240 μs) on the release of mannoprotein. During 25 days of aging, the mannoprotein concentration increased rapidly in all samples treated with PEF. The concentration of mannoprotein

of PEF treated samples was found 6 times higher than the untreated samples (approximate 50 mg/L to 300 mg/L). In addition, after 18 days of aging no significant differences in the mannoprotein concentration were observed in all samples treated with PEF (Martínez et al., 2016). In the current study, a lower amount of yeasts and lower PEF strength were used and thus the amount of mannoprotein released from both PEF treated and untreated were much lower than in the previous study. However, there are similarities in the trend of changes in mannoprotein concentration. For example, the mannoprotein concentration increased until the 32 days of aging and then became constant in the current study, and no significant difference in the mannoprotein concentration was found in the samples treated with different PEF strength. The finding suggests that the function of PEF treatment is generating electroporation which facilitates the contact of the enzyme with the mannoprotein located in the outmost layers of the yeast cell wall.

Martínez et al. (2017) studied factors influencing autolysis of yeast cells treated by a pulsed electric field. The release of mannoprotein from the untreated yeast cells was delayed in the medium containing 10% ethanol (v/v) and pH 3.5, and the impact of PEF treatment (3 µs of 25 kV/cm with a frequency of 0.5 Hz) on the release of mannoprotein was also reduced under such condition. This suggests that the release of mannoprotein in the current study can be limited by wine-like condition. Even though, PEF treatment potentially improved the release of enzymes from yeast cells, the activity of the released enzyme might be limited by wine condition.

β-Glucan

The concentration of β-Glucan during the aging experiment was estimated using the concentration of glucose after acid hydrolysis. Similar to the concentration of mannoprotein, lees material was the major factor affecting the release of β-Glucan during aging of the model wine ($P < 0.05$). For example, the highest concentration of β-Glucan was found in the model wine containing inactivated yeasts (19.0 mg/L) on Day 56, followed by yeasts treated by low PEF strength (approximate 5.6 mg/L), yeasts treated by high PEF strength (approximate 4.8 mg/L) and control (approximate 4.0 mg/L). In addition, analysis of variance demonstrated that PEF strength, and its interaction with aging time and β-glucanase had a significant impact on β-Glucan concentration in model wine ($P < 0.05$) (Table 5.15). However, the interaction between time and enzyme had no impact on β-Glucan concentration ($P > 0.05$). The results were not reported in previous studies.

Table 5.15 The results of the ANOVA analysis of β-Glucan concentration in model wine containing PEF and enzyme induced yeasts.

Source of variation	F probability
Block.Subject stratum	
PEF strength	< 0.001
Enzyme	0.011
PEF strength.Enzyme	0.021
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	0.002
Time.Enzyme	0.060
Time.PEF strength.Enzyme	0.021

No significant difference was found between the β-glucan concentration of PL0 and PL7, and between PH0 and PH7 (P >0.05). Therefore, only the changes in the β-glucan concentration of NR7, PL7 and PH7 were shown in Figure 5.15. In general, the concentration of β-glucan into the model wine increased drastically in the all samples during the first 32 days of aging. On Day 32, the highest concentration of β-glucan was found in PL7 (7.11 mg/L), which is significantly higher than PH7 (6.18 mg/L) and NR7 (4.35 mg/L) (P < 0.05). From Day 32 to 56, the β-glucan concentration of PL7 and PH7 decreased to 5.61 to 4.23 mg/L, respectively; the β-glucan concentration became stable. As explained earlier, the decrease of the β-glucan concentration can be attributed to that glucan is able to form more unstable compounds susceptible to precipitation (Martínez-Lapuente et al., 2013). In addition, low PEF strength (5.5 kV/cm, 16 µs, 5 Hz) with/without enzyme achieved best results in term of the release of β-glucan.

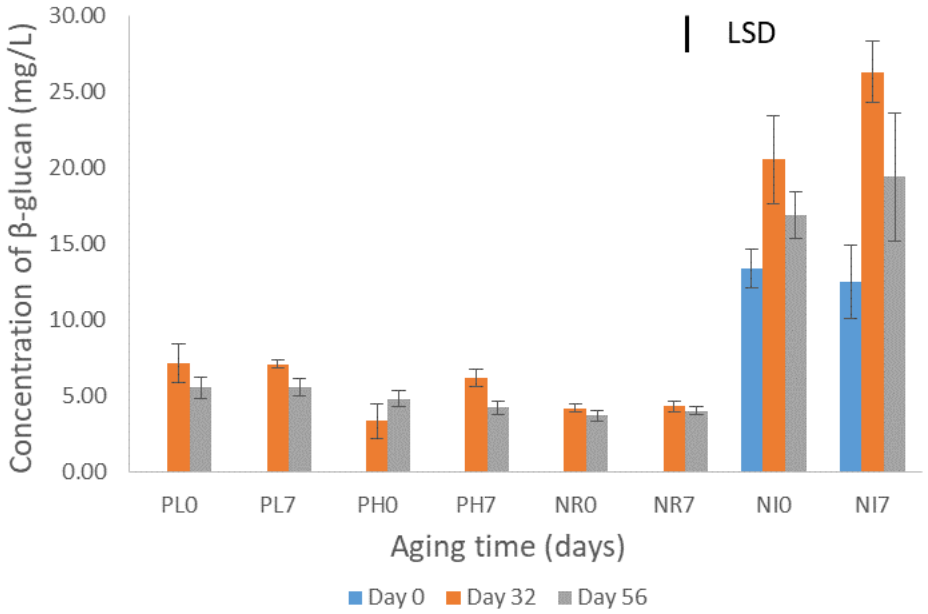


Figure 5.15 Release of β-glucan from different lees materials during aging.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment; NI: inactivated yeasts. 0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

5.4.2.5 Glycerol

The glycerol concentration of the model wine containing different lees material was determined throughout an aging period of 56 days. Analysis of variance was used to study the differences between glycerol concentration due to the lees material, enzyme addition (β-glucanase) and Aging time. Overall, PEF strength, enzyme addition, time and their interactions all had a significant impact on glycerol concentration model wine ($P < 0.05$) (Table 5.16). The information reported in the current study is new regard the changing of glycerol concentration during wine aging.

Table 5.16 The results of the ANOVA analysis of glycerol concentration in model wine.

Source of variation	F probability
Block.Subject stratum	
PEF strength	0.046
Enzyme	< 0.001
PEF strength.Enzyme	0.472
Block.Subject.Time stratum	
Time	< 0.001
Time.PEF strength	< 0.001
Time.Enzyme	< 0.001
Time.PEF strength.Enzyme	0.031

Figure 5.16 demonstrates the changing of glycerol concentration in model wine containing different lees material throughout the aging experiment. In general, the highest glycerol concentration was found in the model wine containing rehydrated yeasts (NR), followed by PEF induced yeasts (PH and PL), and inactivated yeasts (NI). In comparison with aging experiment in Chapter 4, the change of glycerol concentration in model wine containing rehydrated and inactivated yeasts (R-4 and R-ENZ-4) were similar to NR and NI in the current study. The glycerol concentration of PH and PL were very similar (ranged from about 8 to 12 mg/L), and the trend of changing of glycerol concentration in the model wine containing PEF induced yeasts (PH and PL) was similar to the NR.

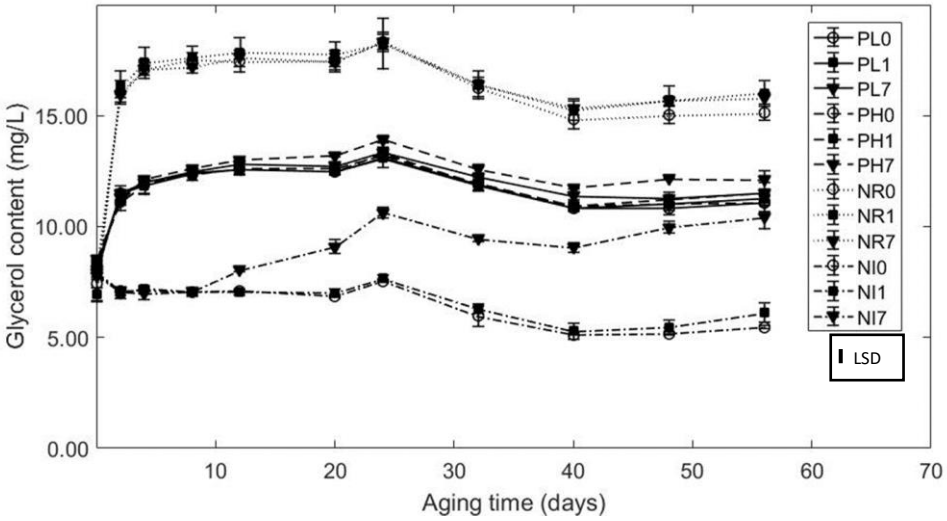


Figure 5.16 Gycerol concentration in the model wine containing different lees material.

PL: 5.5 kV/cm; PH: 10.0 kV/cm; NR: no PEF treatment. 0: no enzyme addition; 1: 1U of β-glucanases; 7: 7.5U of β-glucanases. The error bar in the figure represents the standard deviation of the repeated measurements of all samples (n = 3). The black bar represents the least significant difference (LSD).

In the current study, lees material is a factor which influenced the glycerol concentration which is consistent with the aging experiment in Chapter 4 ($P < 0.05$). During PEF treatment and production of inactivated yeasts, rehydrated yeasts were inactivated, which means these yeast cells no longer produce and accumulate glycerol. NR is the yeast without PEF treatment which might continuously produce glycerol when introduced into the model wine until the beginning of autolysis. Therefore, the highest glycerol concentration was found in NR.

The dosage of enzyme addition significantly affected the released of glycerol concentration from different lees material throughout the aging experiment, especially in the model wine containing NI ($P < 0.05$). The changing of glycerol concentration in model wine with 0U and 1U of the enzyme was similar (Figure 5.16). In contrast, an increase of glycerol concentration in the model wine with enzyme addition of 7.5U started on Day 16 and continued for the rest of the aging experiment. This can be attributed to the enzymatic activity which caused break down of yeast cell membrane and release of glycerol to the model wine.

The PEF treatment and its interaction with enzyme addition were significantly affected the released of glycerol concentration ($P > 0.05$). However, there was no significant difference between the glycerol concentration of PHs and PLs. This might be attributed to the low glycerol concentration in the origin of rehydrated yeasts (less than 18 mg/L) and the detection limit of the analytic instrument.

It is in agreement with the finding in the aging experiment in Chapter 4 that manipulating lees significantly affected the glycerol concentration in the model, but the effects of increase mouthfeel of wine can be very limited. This mainly due to the reported threshold of the perceived viscosity of glycerol is 5 g/L, and the addition of 18 mg/L of glycerol is unlikely to be effective to increase the perceived viscosity.

5.5 General discussion and conclusion

PCA was performed to investigate the overall effects of the PEF treatment (low PEF strength, high PEF strength and control), and enzyme addition (β-glucanase: 0U, 1U and 7.5U) on the chemical compounds released in the model wine after 32 and 56 days of on-lees aging (Figure 5.17 and 5.18).

According to Figure 5.17, the first two principle components explained 64% and 17% of the variance in the dataset, respectively. The variation in the chemical composition of different PEF treatment on Day 32 can be easily distinguished. Most of the samples from the model wine containing PH (both with and without enzyme treatment) had a positive count on both F1 and F2, except PH0. This clearly showed that, on average, the samples from model wine containing PH had concentrations of certain amino acids (including Glu, Ser, Gln, Pro, Arg, Cys, Thr, Asn, Ala, Val, Lys, and Tyr), total free amino acids and protein, and primary amino nitrogen that were above average. All the samples from the model wine containing PL were spread along the negative side of F1; this indicated that the samples from model wine containing PL had concentrations for most chemical compounds that were below average, except acidic polysaccharide and β-glucan. Finally, most of the samples from the model wine containing NR had a positive count on F1 and a negative count on F2; on average, the samples from model wine containing NR had concentrations of certain amino acids (including Met, Phe, Leu, Tyr, Ile, Trp, and Asp) and glycerol that were above average. The result indicated that on Day 32, the chemical composition in the model wine aged on different PEF induced yeasts was different. It is interesting to point out that mannoprotein, neutral polysaccharide and histidine were associated with PL and PH samples; the correlation of these chemical compounds to PL and PH were similar. This might suggest that on Day 32, the PEF treatments used in the current study were able to release more mannoprotein, neutral polysaccharide and histidine compared to the conventional aging techniques. In addition, PEF treatment with high strength was not able to release more of the above chemical compounds than the low strength treatment. However, PEF with the high PEF strength released more of certain amino acids (Glu and Ser) and had a greater primary amino acid nitrogen concentration than with low PEF strength. Moreover, the addition of β-glucanases into the PH samples seem to have some impacts on the chemical composition in the model wine. However, such impacts were very limited in the PL and NR samples.

Figure 5.18 demonstrated the spread of sample datasets on Day 56. The first two principle components explained 37% and 28% of the variance in the dataset, respectively. The general position of the sample datasets were similar to Day 32 with some differences (Figure 5.19). The dataset of PL and PH samples were poorly separated from each other, although the PEF treated samples remained well separated from the NR samples. This clearly indicated that time was a factor affecting the chemical composition of model wine containing PEF treated lees samples rather than PEF strength; the chemical composition in model wine containing the PEF treated yeasts (both high and low strength) were different from the control. The neutral polysaccharide, glutamic acid and serine β-glucan and were found associated with the PEF induced samples.

Enzyme also became a factor affecting chemical composition of the model wine towards the end of aging experiment. For example, PL7 and PH7 were separated from PL0 and PH0; and the addition of 7.5U of β-glucanases favoured the releases of glutamine, histine, lysine and acidic polysaccharide. It is interesting to point out the association between mannoprotein and the samples with the addition of β-glucanases (PL7, PH7, and NR7). This not only showed the addition of 7.5U of β-glucanases favoured the releases of mannoprotein, but also indirectly demonstrated the acceleration of the release of mannoprotein by PEF treatment as the release in conventional aging method caught up at Day 56.

A radar chart was used to demonstrate the effectiveness of PEF treatments for the released chemical compounds (total protein, neutral polysaccharide, Arg, Glu, Pro, and Ala) that may potentially improve wine mouthfeel (Figure 5.19). To avoid the differences due to different concentrations between chemical compounds, as before results were normalized against the average of each compound. The areas of the each lees material represents the effectiveness of the PEF treatments at the end of aging experiment.

The application of PEF treatment (PL0 and PH0) seemed an effective way to improve wine mouthfeel due to their ability of release of the highest amount of neutral polysaccharide and mannoprotein. However, the PEF treatment with a high strength (PH0) might not be effective for the release of β-glucan compare with conventional method (NR0). This can be attributed to the poor stability of β-glucan which might form new chemical structure with the other chemical compounds in the model wine. The addition of β-glucanases can be an alternate option for improving mannoprotein concentration during on lees aging. For example, the higher mannoprotein concentrations were observed in treatment PL7, PH7 and NR7. However, the addition of β-glucanase seems to have negative effects on neutral polysaccharide; the higher dosage of β-glucanase led to the lower neutral polysaccharide concentration. This might be attributed to the precipitation of the fragments of the

polysaccharide. Similar trend was also observed in the concentration of glutamic acid. However there is limited information to explain such observations.

In general, total protein, neutral polysaccharide, mannoprotein, β-glucan were the major chemical compounds which correlated to wine viscosity. Thus, these compounds might be the ones that had contribution to wine mouthfeel. PEF treatment was able to accelerate wine aging and release more mouthfeel related chemical compounds. However, the PEF treatment was still not as effective as the application of inactivated yeasts.

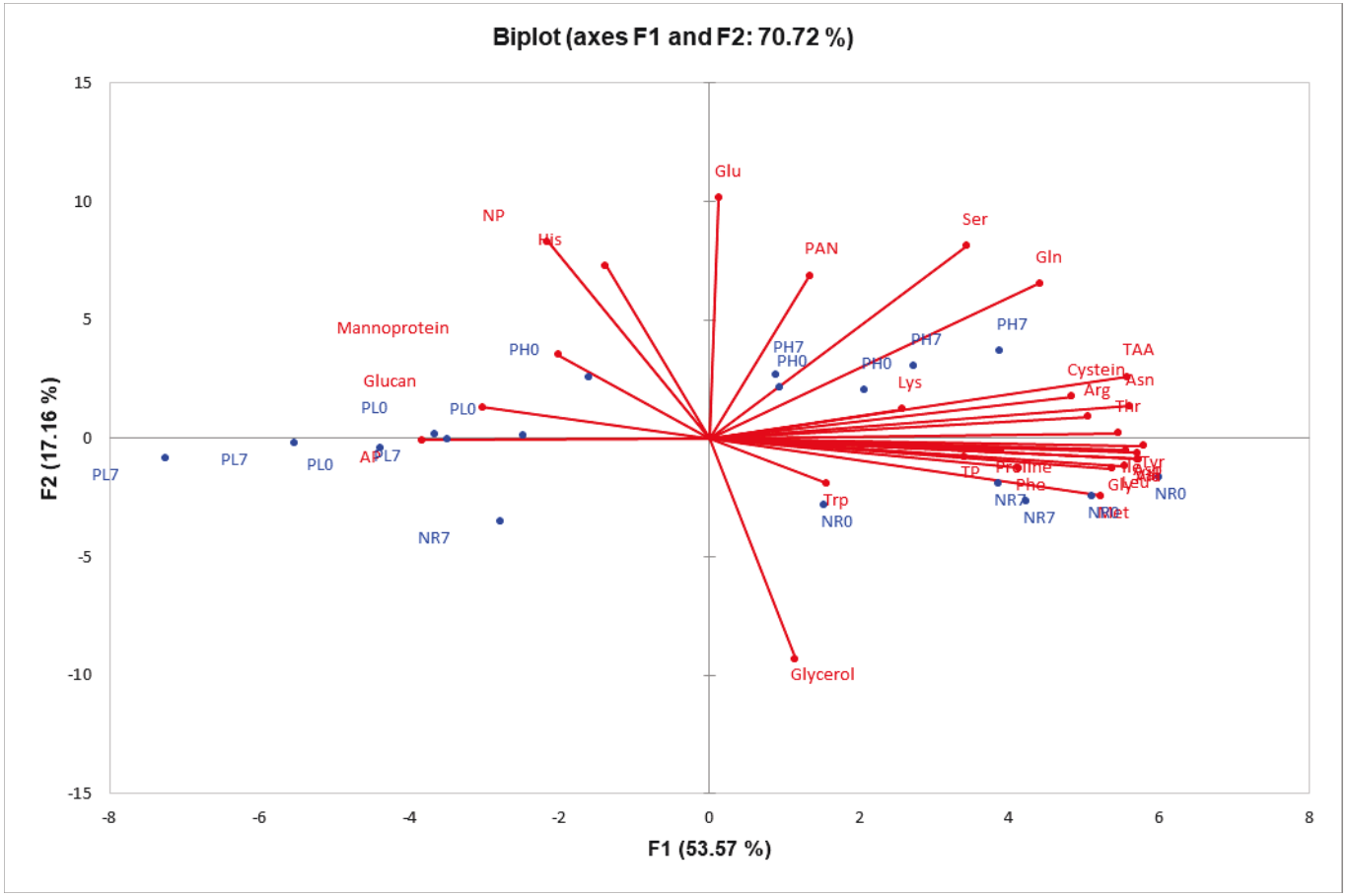


Figure 5.17 Principle component analysis (PCA) biplot of the effect of lees treatments on the chemical composition of the model wines on Day 32. F1 represents a different combination of lees treatments; F2 represents different types of chemical compounds.

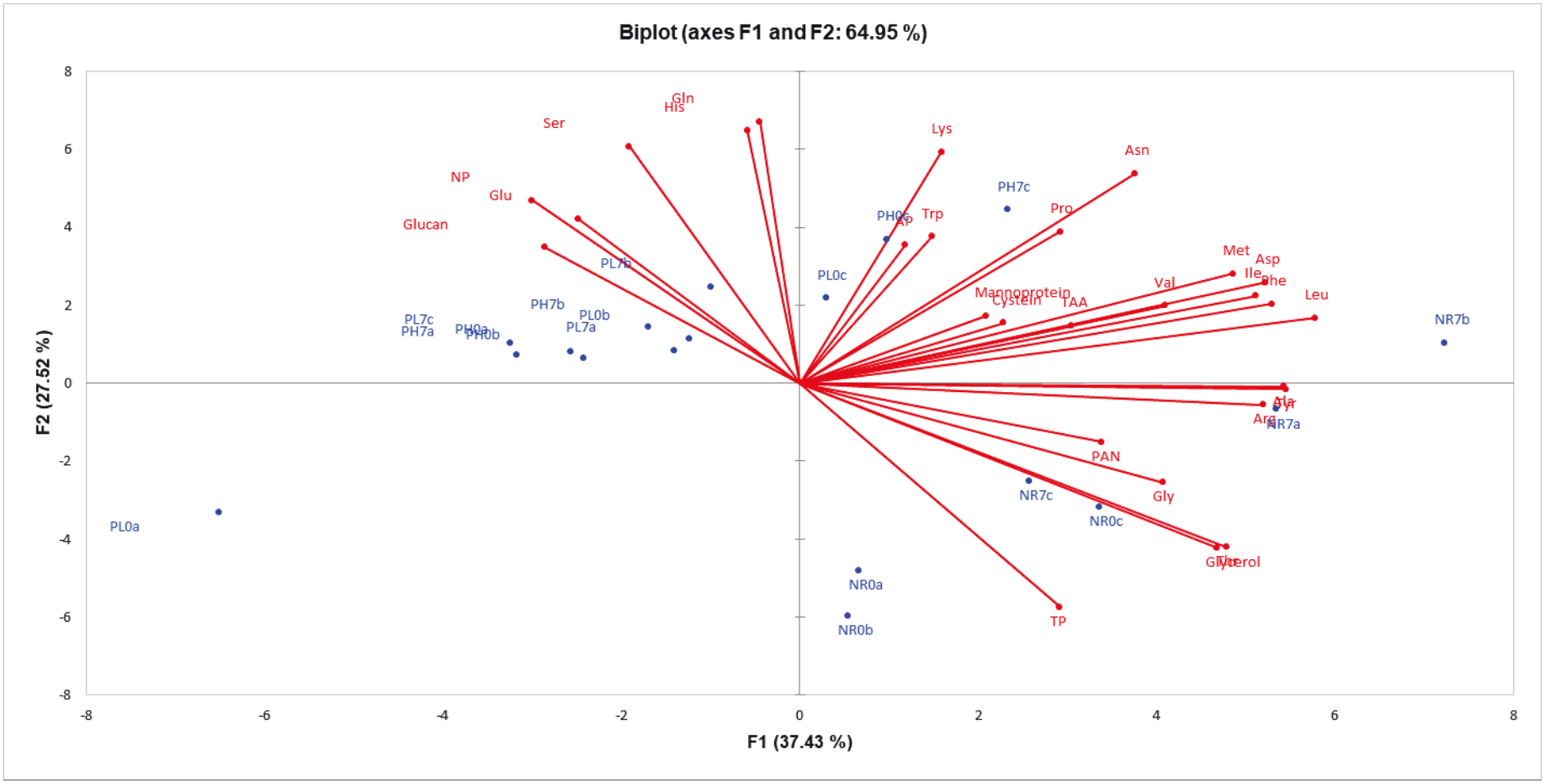


Figure 5.18 Principle component analysis (PCA) biplot of the effect of lees treatments on the chemical composition of the model wines on Day 56. F1 represents a different combination of lees treatments; F2 represents different types of chemical compounds.

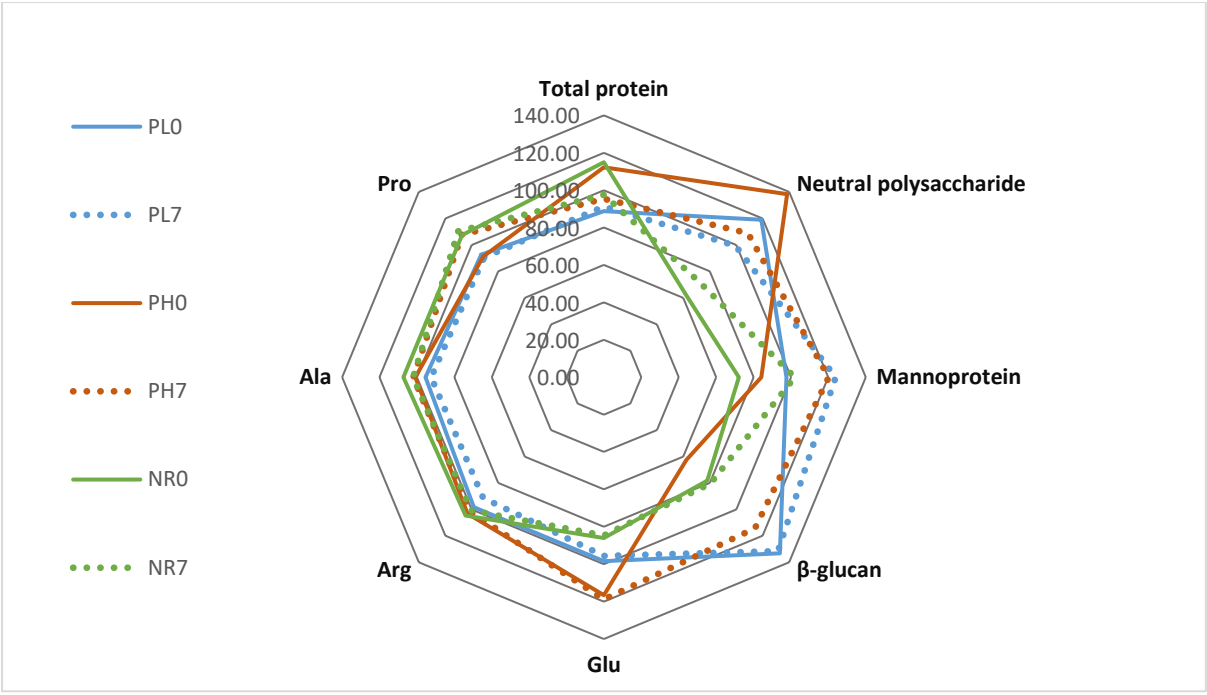


Figure 5.19 The effectiveness of PEF treatments on the release of the chemical compounds which may potentially improve wine mouthfeel.

Chapter 6

Characterization of Commercial Examples of New Zealand Sauvignon blanc Wine

6.1 Introduction

It is well-recognised that contact of lees with wine modifies palate in particular and also aroma. Lees contact is said to impact structure, balance, body and persistence (Escot et al., 2001; Del Barrio-Galán et al., 2011; Fernández et al., 2011; Wang, 2014; Del Barrio-Galán et al., 2011) to the palate; to provide additional aromas and to modify those derived from the grape (Comuzzo et al., 2006; Charpentier & Feuillat, 1993).

However, characterization of sensory attributes related to palate is quite difficult due to lack of an agreed lexicon and supporting scientific data. There is also confusion about words that are used to describe mouthfeel of the wine. For example, bitterness, a taste sensation, is also used to describe the mouthfeel at the end of perception of astringency. Body and weight are also perceptions of mouthfeel, but are used as parameters in sensory analysis separately from other mouthfeel attributes such as astringency. Scientific research had explored the relationship between wine chemical composition and mouthfeel. However, most of the published data is based on red wine.

6.1.1 Napping®

Napping® is a multivariate sensory method in which participants physically place tested products on a large sheet of paper in such a way that the distance between pairs represents the differences between them (Nestrud & Lawless, 2011). It has become an alternative to traditional descriptive analysis (DA) because it is less time consuming but provides similar results (Hopfer & Heymann, 2013). Napping® has been successful in the grouping of alcoholic drinks in recent studies, including Chardonnay wine (Sereni et al., 2016), beer (Giacalone et al., 2016), model wine (Liu et al., 2016), brandy (Louw et al., 2013), and Chenin and Sauvignon blanc wines (Pagès, 2005). In addition, Sereni et al. (2016) has demonstrated the feasibility of using this method for wine mouthfeel assessment. However, no research has utilized this method for characterizing palate attributes in New Zealand Sauvignon blanc wines, especially mouthfeel.

6.1.2 Objectives

The objectives of this study were: to characterize selected Sauvignon blanc wines using napping®; to establish a lexicon of sensory characteristics associated with mouthfeel; and, to link sensory attributes to physical and chemical characteristics of the wines.

6.2 Material and methods

Twenty commercial Sauvignon blanc wines from three different New Zealand wine regions were purchased from local retail outlets in Christchurch, New Zealand to investigate chemical composition (Table 6.1). Eight of these were selected for sensorial evaluation.

Table 6.1. Summary of twenty commercial Sauvignon blanc wines used in this study.

Sample ID	Wine No.[a]	Producer	Wine	Year	Region[b]	Fermentation Vessel	Fermentation Conditions	On-lees treatment	Post-fermentation treatments
J		Jackson Estate	Grey Ghost	2011	M	50% old oak barriques	indigenous yeasts	9 months	
DG	4	Dog Point		2012	M	100% old oak barrels	natural fermentation	in barrel for 18 months	
FB	5	Fairbourne Estate		2013	M	stainless steel, temp controlled, small portion in new oak	neutral yeast		
WM	1	Woollaston Estates	Mahana	2013	N		natural fermentation on skins		in old oak and acacia barrels
MR	2	Millar Road	Green Glow	2013	HB		whole berries, cool temp, indigenous yeast, on-skins for 2-3 weeks, basket press, finished ferment off-skins	6 months	
SW		Catalina Sounds	Sound of White	2013	M	various oak vessels	cultered yeast, warm temp	4 months	mix of large oak foudres and new oak puncheons
SPY1	3	Spy Valley	Envoy, Johnson Vineyard	2013	M	various wood vessels	slow	11 months	in various wood vessels
SPY2				2013	M				
VMSC	6	Villa Maria	Single Vineyard Southern Clays	2013	M		neutral and aromatic yeasts, cool (10-14°C)		
A		Aronui	Single Vineyard	2014	N		aromatic yeast, temp controlled	2 months	
B		Brancott		2014	M		cool temp		
BEA	7	Brancott	Terroir Series	2014	M		cultured yeast, cool temp		small parcel in large oak cuves
G		Giesen		2014	M		selected yeasts, low temp (11-15°C) over 2-3 weeks		
LR		Lime Rock Wines	Coquina	2014	HB	oak barriques and stainless steel	indigenous yeasts	all batches	oak and stainless steel
SC43	8	Saint Clair	Block 3, 43 Degrees	2014	M		selected yeasts, cool temp		
SL		Stoneleigh	Latitude	2014	M	a component in large, old oak cuve	selected aromatic yeasts, low temp		
SC		Saint Clair	Vicar's Choice	2015	M		cool temp, slow		
SLE		Stoneleigh		2015	M		selected aromatic yeasts, cool temp		
VM		Villa Maria	Private Bin	2015	M		cool temp, slow		
PY		Peter Yealands		2015	M		range of selected yeasts, cool temp, long		

a: Wines selected for sensory evaluation (see 6.2.2.1, below); b: Marlborough (M), Nelson (N), Hawke's Bay (HB).

6.2.1 Chemical analyses

Methods used are given in Chapter 3 (Section 3.1). The following analyses were carried out as described: total protein content, primary amino acid nitrogen, free amino acids, neutral and acidic polysaccharides, mannoproteins, glycerol, alcohol, residual sugar, specific gravity and viscosity.

6.2.2 Sensory analysis

The sensory panel comprised fourteen wine professionals primarily oenologists and winemakers and employed in Sauvignon blanc wine production in the Marlborough wine region. There were 5 females and 9 males, with an age range of 33 to 68 years. The panellists were invited via Plant and Food Research, Blenheim, New Zealand. The sensory evaluation study was approved by the Human Ethics Committee of Lincoln University (No. 2015-13). The sensory tests were carried out in the sensory laboratory at Plant and Food Research, 85 Budge Street, Blenheim, on 16th February 2016.

6.2.2.1 Wines and glasses

Eight Sauvignon blanc wines from three different New Zealand wine regions were chosen for their diversity in terms of vinification techniques (see Table 6.1). Two wines had skin contact during winemaking and were from the Nelson (WM) and Hawkes Bay (MR) regions, respectively; the other six wines were from the Marlborough region. In addition, two of these wines (DG and FB, wine numbers 4 and 5, respectively) were randomly selected to be used as control samples during the sensory evaluation. Therefore, in total ten wines were presented to each panellist during the sensory evaluation. For this, 50 mL samples were poured into black, ISO standard tasting glasses (International Organization for Standardization 1977). All glasses were coded with a random three-digit number and covered with watch glasses. Latin sqaure was used to generate the order of the coded glasses for each tasting booth.

6.2.2.3 Napping® and Ultra-flashing profiling.

Napping® and ultra-flashing profiling were used to assess the sensory profiles of commerical examples of New Zealand Sauvignon blanc wines. The sensory evaluation consisted of three parts: Part 1 (Training), Part 2 (Napping 1) and Part 3 (Napping 2). In Part 1, training was given to help panellists practice and become familiar with the napping® technique. Panellists were asked to consider the similarities and/or differences between ten LEGO® pieces (Figure 6.1) according to their own criteria, and to position the LEGO® pieces on the sheet in such a way that pieces were very near to each other if they seemed similar, and distant from one another if they seemed different. The distance between pieces was to indicate their degree of similarity or difference; i.e. more similar pieces were closer, and more different pieces are further apart. After final the placement of all the

LEGO® pieces, the panellists were asked to circle groups of similar LEGO® pieces and write one or more terms which described the reasons the grouping was made. The panellists were encouraged to use as many words as needed.

Figure 6.1 LEGO® pieces used for training of napping® test.

In Part 2, the panellists were required to evaluate ten wines using their skill as wine professionals and the learning in Part 1. Among the ten wines, two had been randomly selected to serve as control treatments. The coded black glasses, including individually coded duplicates, were placed on the table in random order for each panellist. For the evaluation of wines, panellists were asked to consider the similarities and/or differences between the wines in terms of their palate attributes (meaning tastes sweet, acid, bitter, salty, umami), and how the wine felt in the mouth (e.g. astringency, body, viscosity, weight). During the evaluation of wine, panellists were asked to wear a nose clip at all times to eliminate the aroma from retro-nasal olfaction.

In Part 3, the wines were evaluated by the same set of panellists for a second time. The protocol for the sensory evaluation was same as Part 2.

6.2.2.4 Data collection and analysis

Data collection

The position of each glass was determined by measuring the X and Y coordinates of the central point of the bottom of the glass using the bottom left corner of the sheet as the origin. The data were organised as described by Pagés (2016). The descriptive terms for wines and/or wine groups were collected and summarized in a frequency table in three broad categories: flavour (e.g. sweet, salty), mouthfeel (e.g. hot), and other (terms which did not fit into either of these categories). The

frequency table was further divided into three sub-categories representing: descriptor (e.g. oily), quantitative assessment (e.g. low acidity), and expressions that made judgement of characters (e.g. good balance).

Data analysis

Person Performance Index (PPI)

The consistency of each panellist was assessed by calculating a Person Performance Index (PPI) (Hopfer and Heymann, 2013). For each individual map generated by each panellist, the distances between all pairs of glasses was calculated. The PPI was then calculated, being the ratio of the distances between the two duplicate samples and the maximum distance between any non-replicated samples. Values of PPI range between 0 and 1 with the smaller PPI values indicate that panellists placed identical samples closer together (Hopfer and Heymann, 2013).

The conconsistency between Panellists (Alternative method)

An alternative method was also used in the current study to investigate the consistency with which panellists placed the duplicated control samples. This method is reported for the first time in sensory evaluation by napping® and provided a systematic way to create the final consensus map (see Section 6.3.2, below).

Multiple Factor Analysis (MFA)

The data were used to determine a consensus map, this being the result of a MFA (multiple factor analysis) which is performed on the normalized data sets of individual panellists. It demonstrates the global configuration within panellists, and also allows comparisons between individual configurations.

Two of the wines (4 and 5) were control wines and had been introduced as duplicates; ideally these duplicates would have been placed together. If this were the case, then the configuration of the consensus map would remain same if the coordinates of these duplicates were swapped. To test the effect of the nominal labelling system used (i.e. one duplicate of wine 4 and 5 was labelled 4a and 5a, and the other 4b and 5b), a procedure was devised which involved randomly reordering the tabulated coordinates given to the control samples (4a, 4b, 5a, and 5b) for each panellist according to one of the 4 possible combinations. This was repeated 14 times to generate 15 different consensus maps. This procedure was adopted because panellists considered the duplicate control samples as different wines, so it was necessary to investigate the effect on the consensus map of the coordinates given to these duplicate samples.

Microsoft Excel (Microsoft Corporation, 2016) with the XLSTAT-Sensory implementation (2018) was used to perform statistical analyses of the data.

6.3 Results and discussion

6.3.1 Chemical analyses

Table 6.2 summarized the results of physical and chemical characterization of the commercial Sauvignon blanc wines (n=20). Most had alcohol concentrations that varied between 12% and 13%, with the lowest alcohol concentration in sample LR (10.65%). The average viscosity of the samples was 1.72 mPa.s, and the average specific gravity was 0.991. The variation in the viscosity and specific gravity results were very limited.

Greater variation was found in total phenolic concentration. The average total phenolic concentration was 274 mg GAE/L. Most of the samples had total phenolic concentration ranging between 128 and 331 mg GAE/L, except MR (667 mg GAE/L) and WM (562 mg GAE/L), both of which were fermented on skins.

The mean residual sugar concentration was 3.82 g/L, with LR and SW having the greatest and least residual sugar (7.72 g/L, and 1.33 g/L), respectively. The residual sugar concentration in the other samples ranged from 3 to 5 g/L. A similar trend was observed in total polysaccharide concentration, except for VM (7.1 g GALE/L) and MW (1.3 g GALE/L). The majority of polysaccharides in all samples were in neutral form, with only small amounts in acidic form (average of 0.21 g GALE/L). In this study, the concentration of mannose and glucose were used to represent the concentration of mannoprotein and β-glucan, respectively. Mannose concentration in most samples ranged between 80 and 124 mg/L, except J, SPY1 and SPY2. Higher glucose concentrations were observed in SPY1 and SPY2 (117 mg/L), and also in MR (150 mg/L) and DG (116 mg/L).

The total protein concentration in all samples was less than 35 mg BSA/L. Total free amino acids concentration in most samples ranged between 4613 and 6945 µM. The only exception was observed in SC43 with a concentration of 13265 µM. Overall, there is a great variation in chemical composition among the selected wines, which can be attributed to the use of different vinification techniques. This is discussed in more detail below.

Table 6.2 Summary the results of physical and chemical characterization of New Zealand commercial Sauvignon blanc wines.

Sample ID	Alcohol (%)	Viscosity (m.Pa.s)	Specific gravity	Total phenol (mg GAE/L)	Residual sugar (g/L)	Total polysaccharide (g GALE/L)	Neutral polysaccharide (g GALE/L)	Acidic polysaccharide (g GALAE/L)
J	12.9 ± 0.0	1.65 ± 0.01	0.991 ± 0.00	128 ± 4	3.10 ± 0.10	3.3 ± 0.4	3.3 ± 0.5	0.07 ± 0.05
DG	13.0 ± 0.0	1.78 ± 0.05	0.991 ± 0.00	329 ± 1	2.20 ± 0.14	2.1 ± 0.4	2.0 ± 0.4	0.14 ± 0.02
FB	13.2 ± 0.3	1.64 ± 0.01	0.990 ± 0.00	157 ± 2	2.28 ± 0.06	2.0 ± 0.1	1.9 ± 0.1	0.17 ± 0.03
WM	13.1 ± 0.1	1.66 ± 0.01	0.991 ± 0.00	562 ± 8	2.48 ± 0.20	1.3 ± 0.3	1.2 ± 0.3	0.20 ± 0.06
MR	13.1 ± 0.1	1.70 ± 0.03	0.991 ± 0.00	677 ± 18	4.03 ± 0.06	4.3 ± 0.0	3.7 ± 1.0	0.11 ± 0.04
SW	12.6 ± 0.1	1.68 ± 0.01	0.990 ± 0.00	167 ± 9	1.33 ± 0.23	2.0 ± 0.2	1.9 ± 0.2	0.08 ± 0.01
SPY 1	13.0 ± 0.1	1.67 ± 0.01	0.992 ± 0.00	237 ± 16	4.33 ± 0.06	4.2 ± 0.3	4.1 ± 0.3	0.11 ± 0.10
SPY 2	12.7 ± 0.4	1.64 ± 0.00	0.993 ± 0.00	303 ± 13	4.55 ± 0.18	5.9 ± 0.4	5.8 ± 0.3	0.12 ± 0.03
VMSC	12.8 ± 0.8	1.76 ± 0.03	0.992 ± 0.00	229 ± 7	5.48 ± 0.10	6.2 ± 0.1	5.4 ± 0.9	0.27 ± 0.16
A	12.9 ± 0.0	1.77 ± 0.03	0.991 ± 0.00	176 ± 9	2.67 ± 0.06	2.8 ± 0.2	2.7 ± 0.2	0.20 ± 0.02
B	12.6 ± 0.1	1.80 ± 0.00	0.992 ± 0.00	295 ± 10	3.63 ± 0.15	5.2 ± 0.0	5.0 ± 0.1	0.30 ± 0.05
BEA	12.7 ± 0.1	1.68 ± 0.01	0.986 ± 0.00	248 ± 2	3.80 ± 0.26	4.1 ± 0.3	4.0 ± 0.2	0.24 ± 0.03
G	12.2 ± 0.1	1.79 ± 0.01	0.992 ± 0.00	260 ± 7	3.03 ± 0.06	3.6 ± 0.1	3.4 ± 0.1	0.26 ± 0.07
LR	10.7 ± 0.1	1.58 ± 0.01	0.995 ± 0.00	145 ± 3	7.72 ± 0.18	7.3 ± 0.6	7.2 ± 0.7	0.22 ± 0.14
SC43	12.6 ± 0.3	1.61 ± 0.00	0.993 ± 0.00	195 ± 3	4.15 ± 0.07	5.7 ± 0.3	5.5 ± 0.3	0.33 ± 0.04
SL	12.9 ± 0.0	1.74 ± 0.01	0.991 ± 0.00	192 ± 1	3.80 ± 0.00	4.1 ± 0.1	4.0 ± 0.1	0.24 ± 0.09
PY	13.1 ± 0.0	1.79 ± 0.01	0.992 ± 0.00	301 ± 2	4.43 ±0.06	4.8 ± 0.1	4.7 ± 0.0	0.24 ± 0.11
SC	13.0 ± 0.1	1.75 ± 0.02	0.993 ± 0.00	331 ± 5	3.80 ± 0.10	5.6 ± 0.2	5.4 ± 0.2	0.25 ± 0.04
SLE	12.9 ± 0.3	1.84 ± 0.02	0.991 ± 0.00	250 ± 6	4.47 ± 0.06	5.2 ± 0.1	5.0 ± 0.2	0.25 ± 0.18
VM	13.0 ± 0.1	1.83 ± 0.01	0.992 ± 0.00	298 ± 4	5.17 ± 0.06	7.1 ± 0.4	6.9 ± 0.4	0.34 ± 0.15

Note: g GAE/L: g galactose equivalent/L; g GALAE/L: g galacturic acid equivalent/L; mg BSA/L: mg bovine serum albumin/L.

Table 6.2 (continued).

Sample ID	Glycerol (µmol/L)	Total protein (mg BSA/L)	Mannose (mg/L)	Glucose (mg/L)	Total free amino acids (µM)
J	1154 ± 3	11.7 ± 0.2	140	34	3878
DG	464 ± 13	25.7 ± 0.8	124	116	5864
FB	720 ± 2	10.3 ± 0.2	71	34	3733
WM	661 ± 9	21.0 ± 0.6	120	61	3662
MR	1027 ± 31	35.0 ± 1.2	108	150	2667
SW	909 ± 14	11.5 ± 0.1	112	44	5290
SPY 1	379 ± 7	12.4 ± 0.1	152	117	5380
SPY 2	429 ± 4	10.7 ± 0.4	158	117	4966
VMSC	779 ± 13	16.3 ± 0.2	100	41	6305
A	886 ± 8	12.8 ± 0.5	65	62	4994
B	813 ± 17	10.4 ± 0.2	131	56	7157
BEA	714 ± 16	10.1 ± 0.3	110	43	7606
G	1240 ± 36	8.7 ± 1.0	118	30	6309
LR	616 ± 3	16.4 ± 0.3	94	64	4496
SC43	652 ± 26	10.5 ± 0.0	79	52	13265
SL	610 ± 7	11.1 ± 0.3	88	63	5212
PY	689 ± 7	9.2 ± 0.3	61	58	5858
SC	908 ± 14	8.8 ± 0.3	82	36	8882
SLE	491 ± 6	8.7 ± 0.2	91	69	5046
VM	889 ± 30	10.1 ± 0.2	75	41	7775

.

PCA was performed to further investigate the variability in chemical composition of the surveyed Sauvignon blanc wines (n = 20) (Figure 6.2). The first two principle components explained 31% and 22% of the variance in the dataset, respectively. Broadly speaking, the distribution of wines on the biplot gave rise to two main groupings: those mainly clustered in the upper and lower left quadrants, and those in the lower right quadrant.

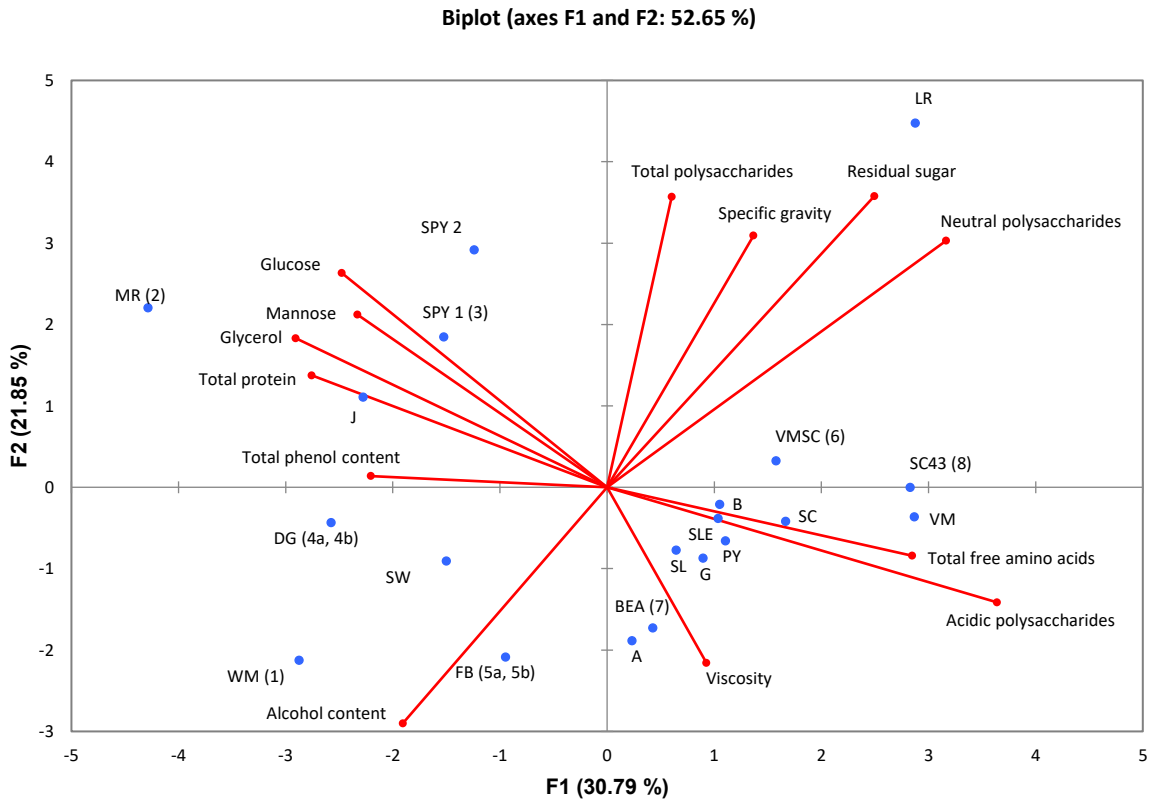


Figure 6.2 Principle component analysis (PCA) of the chemical composition of the surveyed Sauvignon blanc wines. The number in the bracket corresponds to the wine number in the sensorial evaluation (Table 6.1).

Refering to Table 6.1, the wines in the upper left quadrant were somewhat older and had mostly undergone lees aging in the range 4 to 11 months. The vectors associated with this quadrant were those for glucose, mannose, glycerol, and total protein. Glucose and mannose in these wines can be attributed to the release of mannoprotein during on-lees aging; and the release of mannoprotein also made a contribution to the total protein concentration. Furthermore, the wines in the upper and lower left quadrant had some association with total phenol concentration. Two explanations are apparent: first, MR and WM were fermented on skins; second, wines SPY (1 and 2), J, DG, and SW or

their portion were fermented and/or aged in oak barrels with J being fermented at a warm temperature. These vinification techniques enhance the extraction of phenolics to wines. This might also allow better extraction for phenolic compounds.

Wines FB, BEA, A, and all the wines in the lower right quadrant were fermented in stainless steel tanks, although wines FB and BEA contained small portions that were fermented in oak barrels. Wine FB had highest alcohol concentration among the selected wines. Wine A had been aged on-lees aging for two month with weekly stirrings. In addition, these wines in the lower right quadrant formed a cluster associated with the vector for total free amino acids. That these wines had a higher total free amino acids concentration compared to those with on-lees aging was not expected, because on-lees aged wines might be expected to have a higher total free amino acids concentration due to the release and hydrolysis of nitrogenous compounds from yeast cells. It should be noted that compairisons of total free amino acids concentration in previous studies were between 'aged wines' and 'their control or base wines'. The wines surveyed in the current study were from different wineries and produced by different vinification techniques. For example, wine SC43, G, SLE, P, VM and A were fermented by selected yeasts, which were not specified in the other wines. According to the findings in the current study, lees material is a major factor that can affect total amino acids concentration. In addition, deamination of amino acids and the formation of different compounds during on-lees aging might reduce total amino acids concentrations (Martínez-Rodríguez et al., 2002). Finally, LR had the highest values for residual sugar, specific gravity, total and neutral polysaccharide and was located in the upper right quadrant, associated with the vectors for these parameters.

6.3.2 Sensory analysis

6.3.2.1 Investigation of consensus maps resulting from random re-labelling of control wines

Fifteen consensus maps were calculated as described above. Figure 6.3 demonstrates four examples selected to illustrate the range of the results obtained. For the consensus maps, the first two principal components together explained 35 % to 36 % of the variance in the data sets. Re-ordering of the coordinates of the control samples had no major effect on the results. In more detail, even though the positions of the control replicates were changed, the relative position of wine 1, 2, 3, 6, 7, and 8 on the consensus maps remained relatively unchanged. For example, in all observations, wines 8 and 1 were always located in the top left and right quadrant, respectively; and wines 2 and 3 were always found in the left and right quadrant at the bottom, respectively (Figure 6.3). The positions of other wines were slightly changed along one of the axis. For example, the position of wine 6 changed from positive side of F1 in Observation A to negative side in Observation D; and wine

4a changed from negative side of F2 to positive side. The results indicate that the random re-labelling of duplicate control wines did not change the resulting consensus maps significantly. All the maps were valid, and one consensus map was selected for further analysis with the descriptors.

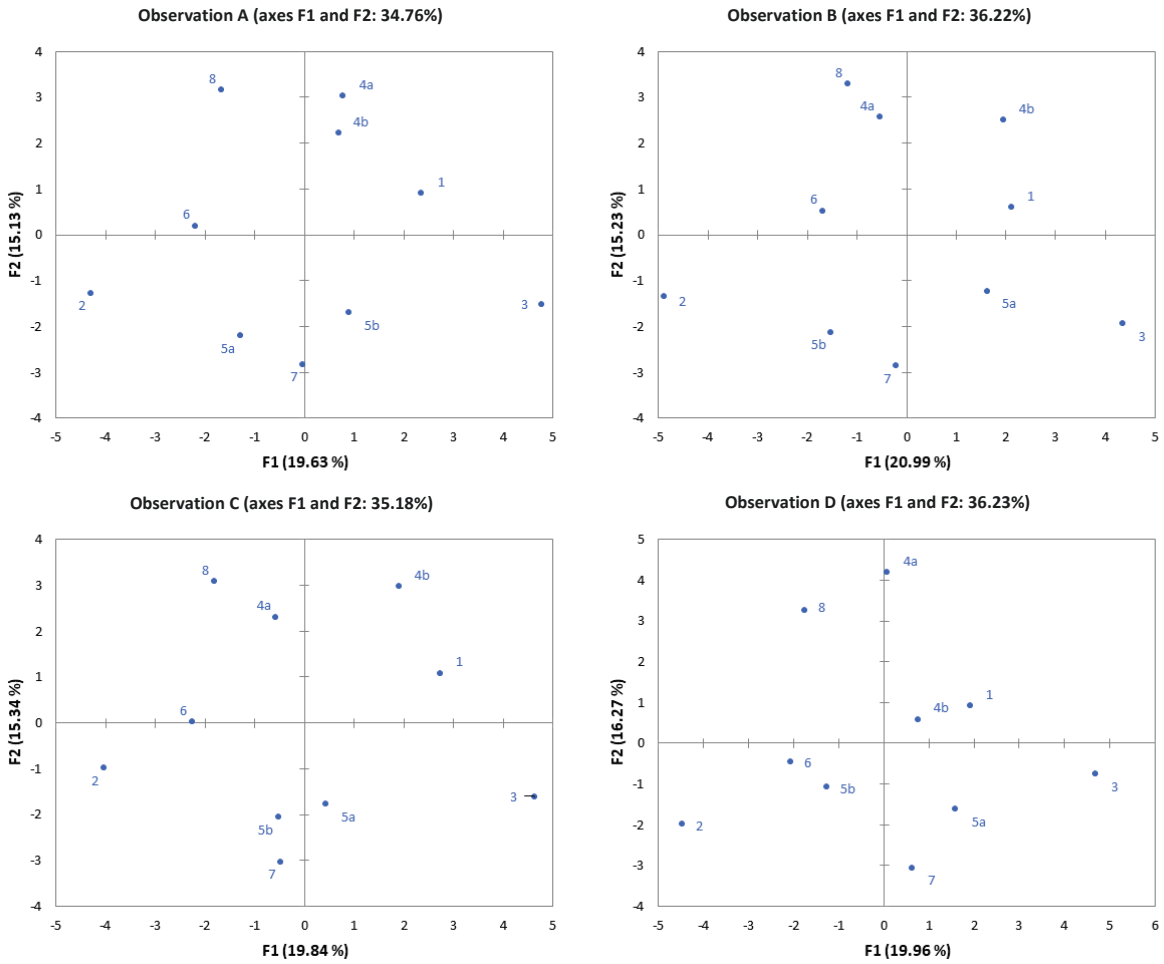


Figure 6.3 The representation of the consensus maps (Observation A - D) based on the generated data sets.

6.3.2.2 Selection of a single representative consensus map

A procedure was devised in order to make the selection of the consensus map for further analysis with the descriptors. The scores of Factor 1 and 2 were used as coordinates, and the distances between the consensus positions of the different wines in each of the 15 maps, was used to calculate the mean and standard deviation of the distance between each pair of wines (Table 6.3). The consensus map with the maximum count for distances within the range (average ± standard deviation) between all pairs of wines was selected.

Table 6.3 Arrangement for the distances between wines in different consensus map.

Consensus map No.	D:1-2[a]	…	D:7-8
1		…	
…….		…	
8		…	
9		…	
…….		…	
15		…	
Mean		…	
Standard deviation		…	

[a] Represents the distance between wine 1 and 2 on consensus maps

This count number varied between 21 and 42 (out of a possible maximum of 45) and was highest for the following map (Figure 6.4). For this map, the first two principle components explained 19 % and 15 % of the variance in the data sets, respectively.

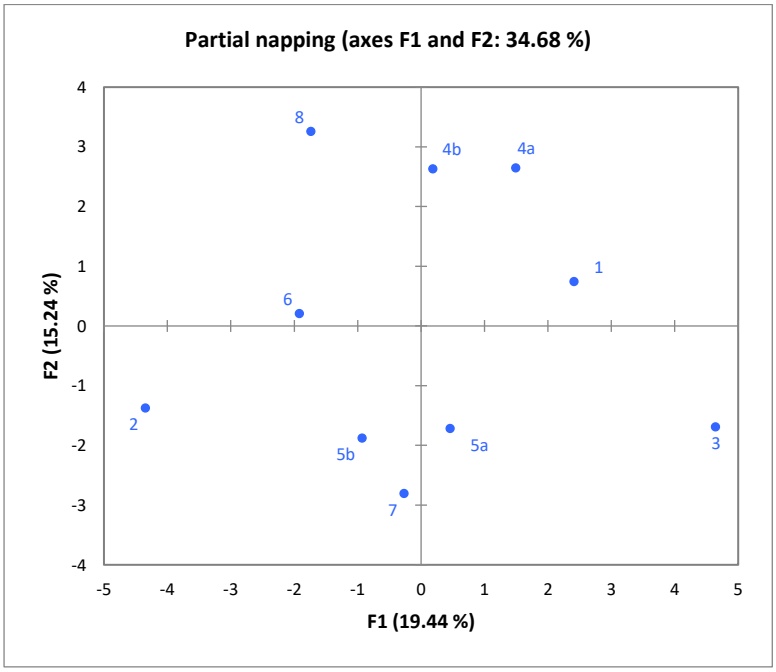


Figure 6.4 Consensus map selected showing the scores the wines used for further analysis with descriptors.

In previous napping® studies on Chardonnay wine (Sereni et al., 2016), beer (Giacalone et al., 2016),

model wine (Liu et al., 2016), brandy (Louw et al., 2013), and Chenin and Sauvignon wines (Pagès, 2005), the first two dimensions combined explained 33% to 66% of the total variance in the data sets. In comparison, the total variance explained by the first two principle components in the current study was at the lower end of this range, similar to the study of Chardonnay wines. In that study on the mouthfeel of Chardonnay wines, Sereni et al. (2016) demonstrated that the volatile fraction was involved in establishing relationships between chemical composition and mouthfeel perception. Despite the types of products, the number of samples, the number of panellists and the training methods, wearing of nose pegs might have created difficulties to panellists for mouthfeel perception as wine aromas were excluded in the sensory characterization.

6.3.2.3 Panellists' performance as measured by RV coefficient.

The RV coefficient is a multivariate generalization of the squared Pearson correlation coefficient and varies between 0 and 1 depending on the variance shared by two matrices. The greater the RV coefficient, the stronger the correlation between the maps of the panellists. On the other hand, low RV coefficients suggest that panellists use different criteria to determine the overall degree of similarity between the samples (Risvik et al., 1997). Table 6.4 summarizes the RV coefficients of 14 panellists for the selected map.

The mean and standard deviation of RV coefficients was 0.624 ± 0.065. Seven panellists had an RV coefficient greater than 0.500 but less than 0.600; the rest of the panellists had an RV coefficient greater than 0.600, and the highest coefficients were for panellists C, F, J, and M. In previous studies, RV coefficients were often used as a metric to exclude panellists that had weak correlations with the other panellists (Giacalone et al., 2016; Liu et al., 2016; Pagès, 2005; Torri et al., 2013; Vidal et al., 2014). RV coefficients of the panellists were reported in some of the studies. Vidal et al. (2014) conducted a full napping study to characterize ten red wine samples using 47 consumers; the mean RV coefficients ranged from 0.5 ± 0.1 to 0.7 ± 0.1. In a partial napping study of nine beers with novices (n = 9) and experts (n =8), Giacalone et al. (2013) found that the mean RV coefficients of novices and experts were 0.405 ± 0.169 and 0.618 ± 0.167, respectively. Despite the differences between wines, the number of samples and the number of panellists, the mean RV coefficients of the napping in the current study fell into the range that was reported in the previous studies. It was concluded that in the current study there was a strong correlation between the maps of all panellists, and therefore no panellists should be excluded from data set.

Table 6.4 Summary of RV coefficients and person performance index (PPI) of 14 panellists.

		Replicate 1		Replicate 2		
Panellist	**Selected map**	**PPI1[a]**	**PPI2[b]**	**PPI1[a]**	**PPI2[b]**	**Overall average**
A	0.548	0.52	0.35	0.15	0.34	0.34
B	0.630	0.87	0.37	0.64	0.53	0.60
C	0.735	0.77	0.22	0.40	0.30	0.42
D	0.586	0.35	0.43	0.34	0.86	0.49
E	0.596	0.23	0.76	0.27	0.47	0.43
F	0.674	0.99	0.62	0.18	0.23	0.51
G	0.638	0.94	0.13	0.35	0.49	0.48
H	0.556	0.32	0.73	0.51	0.67	0.56
I	0.592	0.16	0.44	0.38	0.69	0.42
J	0.701	0.15	0.98	0.19	0.39	0.43
K	0.542	0.12	0.15	0.35	0.77	0.35
L	0.554	0.38	0.47	0.67	0.11	0.41
M	0.702	0.69	0.13	0.26	0.26	0.34
N	0.676	0.47	0.43	0.29	0.29	0.37
Mean	0.624	0.50	0.44	0.36	0.46	0.44
SD	0.065	0.31	0.26	0.16	0.22	0.06

[a] PPI1, PPI calculated for the wine sample No. 4; [b] PPI2, PPI calculated for the wine sample No. 5.

6.3.2.4 Person Performance Index (PPI)

The Person Performance Indices (PPI) for all panellists are presented in Table 6.4. PPI1 and PPI2 represent the PPI based on the two selected control wines (4 and 5), respectively.

The mean scores of PPI1 and PPI2 of all panellists in the two replicate napping experiments were 0.50 ± 0.36 and 0.44 ± 0.26, and 0.36 ± 0.16 and 0.46 ± 0.22, respectively. In the first experiment, four panellists, E (0.23), I (0.16), J (0.15) and K (0.12) who had a low PPI1 score (PPI < 0.30). There were also four panellists, C (0.22), G (0.13), K (0.15) and M (0.13) with a low PPI2 score. In the second experiment there were six panellists, A (0.15), E (0.27), J (0.19), L (0.18), M (0.26) and N (0.29) who had a low PPI1 score (PPI < 0.30). There were also five panellists, C (0.30), F (0.23), L (0.11), M (0.26) and N (0.29) that had a low PPI2 score. This demonstrated that most of the panellists had relatively good performance either for PPI1 or PPI2; panellists C, E, J, and M had the highest degree of consistency. In addition, the overall average PPI scores of all panellists were 0.44 ± 0.06. This indicated that the overall performance of all panellists was not significantly different.

The results also showed that the overall performance of all panellists in the second experiment was not significantly better than in the first. Such a result is similar to Hopfer and Heyman (2013) for

which most panellists had no improvement over replicate sessions. These workers noted that the replicated samples in a napping experiment are a good tool to access the performances of the panellists, but that guidelines are needed in order to identify panellists with 'good performance'.

6.3.2.5 Differences between panellists

Figure 6.5 illustrates the scores of each panellist for the first two principle components of the selected consensus map. Differences between some panellists can be distinguished. In general, panellists can be divided into two main groups and suggested that the placement of the wine samples for panellist G, K and M were different from the rest of panellists (circled in red).

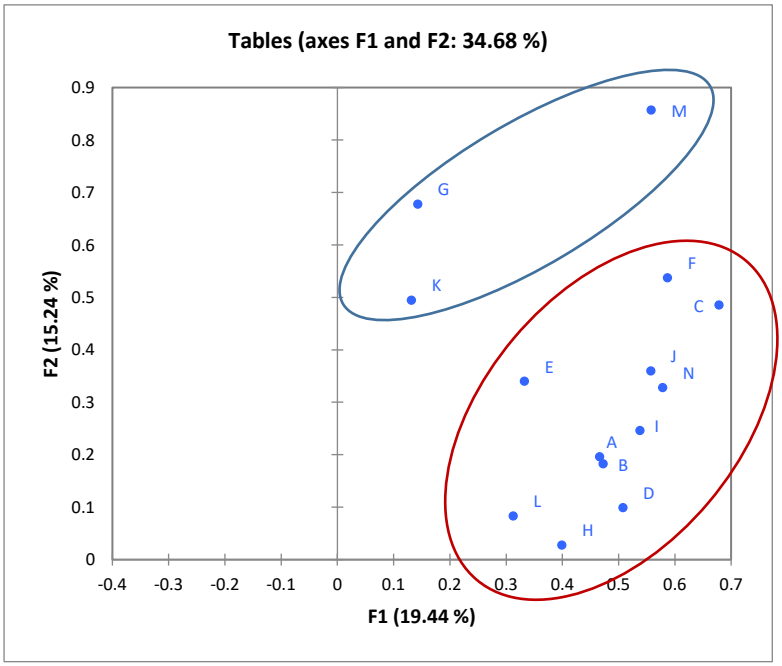


Figure 6.5 Consensus map selected showing scores for individual panellists.

6.3.2.6 Descriptors

Initial reduction in the number of descriptors

In the napping experiment a total of 253 descriptors were collected. These were classified into four broad (flavour, mouthfeel, flavour/mouthfeel, miscellaneous) and a number of minor categories (Table 6.5). The descriptors were further classified in minor category. For example, 'tropical', 'slight green' and 'rounded palate weight' were classified as 'fruity', 'herbaceous & vegetative' and 'weight' minor categories, respectively. In addition, all descriptors were categorised to indicate whether they were descriptive, quality or judgement terms.

The number of descriptive, quality and judgement terms were 130, 94, and 28, respectively. The

majority of the descriptors were used no more than four times on different occasions (Figure 6.6). For example, about 80 and 60 different descriptors were only used once and twice, respectively, on different occasions. Only a few descriptors were used five times and more. Therefore, synonyms and near-synonyms were combined to reduce the number of the descriptors. This combining of terms was done between descriptors within the same categories and sub-categories only; and no combination was done between descriptive, quality and judgement terms. After combination, the frequency distribution of descriptors changed as expected and the total number of descriptors was reduced to 218 (Figure 6.6). In more detail, the numbers of descriptive, qualitative and judgement terms were 111, 81, and 26, respectively. However, the reduction of the number of descriptors was very limited as there were still a lot of descriptors that were only used once or twice.

Table 6.5 Example of classification of the collected descriptor in sensory Napping®.

Descriptors	Broad Category	Frequency	Minor Category	Descriptor	Quality	Judgement
Juicy	M	4		D		
Tropical	F	9	Fruity	D		
Reasonable tropical fruit flavours in the	F	2	Fruity			J
Very intense through out the palate	M	2	Intensity		Q	
Rounded palate weight	M	1	Weight	D		
Slight green	F	4	Herbaceous & vegetative		Q	
Some greeness	F	1	Herbaceous & vegetative		Q	
Average green	F	2	Herbaceous & vegetative		Q	
Green	F	12	Herbaceous & vegetative	D		
Greener notes	F	1	Herbaceous & vegetative		Q	
Greener charactors behind	F	2	Herbaceous & vegetative		Q	
Phenolic	M	6	Woody/Astringent	D		
Slight phenolic finish	M	1	Woody/Astringent		Q	
Mix of green and ripe flavour	F	1	Herbaceous & vegetative	D		
Looking at ripe spectrum	F	1	Fruity	D		
Starting to move into ripe spectrum	F	1	Fruity			J
Riper flavour profile	F	2	Fruity		Q	

Note: F: Flavour; M: Mouthfeel; D: descriptive; Q: qualitative; J: judgement.

In a napping study on beers, Giacalone et al. (2013) relied on descriptors having frequency of more than 20. Thus, in order to reduce the number of descriptors to a manageable number, in the current study descriptors with a frequency no less than 5 were selected, instead of using the descriptors after combination.

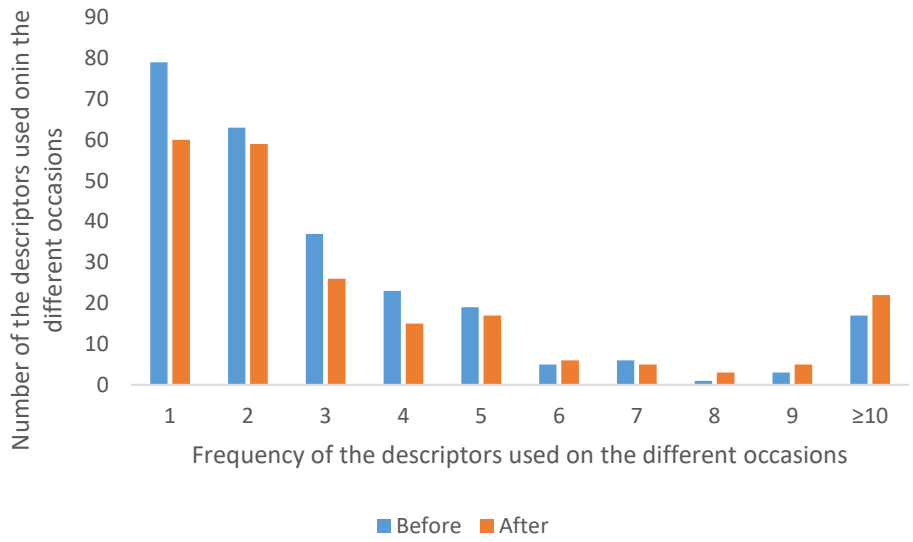


Figure 6.6 Frequency of the descriptors used on the different occasions before and after reduction.

6.3.2.7 Representation of the sensory descriptors in relation to the consensus map

The selected sensory descriptors (n = 48) were overlaid on the consensus map (Figure 6.7). The size and colour of font illustrate the frequency of use. The large bold font in red, the large font in green, and the small font in blue and in black represent frequency of use that ≥ 40, ≥ 20, ≥ 10 and ≥ 5 times, respectively. Descriptors used no more than 5 times have been omitted.

The word 'sweet' was used the most often (≥ 40 times), and it was close to the centre of the consensus map. The words 'hot', 'salty', 'dry' and 'astringent' were also important descriptors which had a frequency of use no less than 20 times. These descriptors together with 'sweet' appeared to form a new axis which is represented by an orange dotted line (Figure 6.7). The words 'sweet' and 'dry' were located at opposite ends of this axis, and these locations were also consistent with the positions of the descriptors for acidity, where 'low acidity' and 'high acidity and/or acid' corresponded to the direction of the line of 'sweet' and 'dry'.

'Not astringent' and 'astringent' were also located at opposite ends of this axis. This might be the result of confusion of the panellists between 'astringent' and 'acidity'. In a study of the mouthfeel of model wine with no phenolic substances, Gawel et al. (2016) demonstrated that the intensity of perceived acidity and astringency was strongly correlated. In addition, the concentration of phenolic compounds in white wines are usually low. A similar trend was also found with descriptors 'short' and 'back palate long' or 'good length'. In addition, the descriptors 'alcohol' and 'hot' were located near the word 'sweet': perceived hotness of a white wine can be attributed to the ethanol content (Gawel et al., 2017), and alcohols, such as ethanol and glycerol, also contribute sweetness in the

final wine (Bakker and Clarke, 2011).

Figure 6.7 Representation of sensory descriptors overlaid on the consensus map. Terms in large fonts and colour were used more frequently (large bold font in red: > 40 times; large font in green: > 20 times; small font in blue: > 10; small font in black: .5). Descriptors used no more than 5 times have been omitted.

Inspection of Figures 6.2 and 6.7, suggested that there was no clear correlation between the panellists' perception and chemical composition of the wines. For example, wines 2 were 3 associated with the vectors for total protein, glucose, mannose and glycerol concentrations. However, wines 2 and 3 were located at the two ends of the new axis in Figure 6.7. In addition, wines 8 and 3 had a similar residual sugar concentration (4.15 and 4.35 g/L), but these wines were perceived as 'sweet' and 'dry', respectively. These apparent inconsistencies can likely be attributed

to the complexity of wine composition and interaction between different perceptions.

On the basis of their chemical composition, all the selected wines can be considered as dry; 'sweetness' from residual sugar should not be perceived as its concentration was lower than the detection threshold (glucose: 11.7 g/L) (Bakker and Clarke, 2011). Perceived sweetness can be attributed to the presence of ethanol and glycerol (as well as, in normal circumstances, 'sweet' volatile aroma compounds). In dry wine, glycerol contributes a slightly sweet taste, and ethanol is able to enhance the sweetness of sugars (Jackson, 2008). Furthermore, the perception of sweetness is modified by the balance between sugars and acids, as sugar is able to suppress sourness. In the current study, this balance occurs mainly between alcohol and acidity, as the residual sugar level is low in dry wines (Peynaud, 1997). This effect can be one of reasons for the perception of different levels of acidity; hence, the descriptors 'low acidity' and 'high acidity and/or acid' corresponded to the direction of the line of 'sweet' and 'dry'.

Phenolic compounds are responsible for astringent and bitter sensations (Jackson, 2008). Wine 3 was associated ith the descriptor 'astringent' by panellists which had a similar or lower total phenolic concentration (237 mg GAE/L) than than the rest of the wines along the new axis. In fact, the highest total phenolic concentration (677 mg GAE/L) was found in wine 2 which was associated with the descriptor 'not astringent' along with wine 4 and 6. Phenolic compounds are also responsible for bitterness (Jackson, 2002). Wines 2, 6, and 4 were associated with 'bitter', 'bitterness' and 'slightly bitter'. Interestingly, wine 3 was not associated with 'bitterness', although it had a similar total phenolic concentration as wine 6. Two possible explanations are: first, sugars and ethanol might suppressed perceived astringency and bitterness; and second, mannoproteins might suppressed perceived bitterness. Vidal et al. (2004) suggested that the mixture of mannoprotein and arabinogalactgan proteins were able to inhibit bitterness of polymeric flavnols extracted from grape seeds.

Although it was difficult to associate the consensus map with chemical composition of the selected wines, an attempt was made to draw on the information concerning vinification techniques for the selected wine (Table 6.1).

In Figure 6.7, wines 6 and 8 were located in the top left quadrant. Both of these wines were made using no (or minimal) skin contact, fermentation in stainless steel tank with commercial yeasts, and no on-lees aging, typical for the majority of Sauvignon blanc produced in Marlborough. The was an association between these wines and various descriptors including 'green', 'short', 'boxwood', and 'tropical', often used in descriptions of a typical Marborough Sauvignon blanc. In contrast, wines 1

and 4 were located in the top right quadrant. These wines differed from the 'typical' Marlborough Sauvignon blanc wine (6 and 8) because fermentation occurred on skins (wine 1) or there was a long period (18 months) of on-lees aging (wine 4). The other wines (2, 3, 5 and 7) were position in the lower two quadrants. Wine 3 was located in the most extreme position in the lower left quadrant, and its production involved fermentation in various wood vessels as well as an extended period (11 months) of on-lees aging. The other wines also represented significant variation in production from 'typical' Marlborough Sauvignon blanc: wine 2 was from Hawkes Bay (which is known to produce wines that differ stylistically from those of Marlborough), was fermented on skins and had 6 months on-lees; wine 5, involved both 6-7 months of on-lees aging and a portion fermented in new oak; and wine 7, which involved maturation of a small parcel in large oak cuves. One interpretation of Figure 6.7, therefore, could envisage so-called 'typical Marlbourough Savignon blanc located in the upper left quadrant, with wines that deviate significantly from these occupying positions in the other quadrants depending on the extent and/or type of deviation from standard practice.

6.4 Conclusion

To conclude, napping® successfully distinguished the differences and similarity of the selected Sauvignon blanc wines. Even though panellists were asked to sort the wines based on the mouthfeel, 'sweetness' was used as the major criteria when sorting the wines; and 'acidity', 'bitterness', and 'astringency' were used as the secondary criteria. The establishment of a lexicon to define mouthfeel was not successful as too many descriptors with a low frequency were produced. There was a poor association between the results of the napping exercise and chemical composition, likely due to the complexity of the interactions between different perceptions. However, the sensory evaluation seemed to be indicate that perceptions were influcenced by vinification, mainly on-lees aging and use of oak.

Chapter 7

General discussion and conclusion

Many studies have demonstrated the release of chemical compounds during wine aging on lees, and have shown improved mouthfeel in the resultant wines (Del Barrio-Galán et al., 2011; Ribeiro et al., 2014; Wang, 2014). In these studies, different yeasts or lees materials, with or without periodic stirring and enzyme addition, were utilised to improve the sensory aspects of the wine. However, individual studies have investigated only one or two of these factors. The present study aimed to provide more comprehensive information by including all of the factors in a multi-factorial trial in order to ascertain the relative importance of each factor (and any resulting interactions) on a range of compositional variables known to influence sensory attributes related to mouthfeel. Furthermore, in order to understand the significance of changes in composition, it was felt that a better appreciation of the relationship between composition and mouthfeel-related sensory attributes was required.

Specifically, the objectives of this study were: 1) to investigate the effects of different lees management techniques on the release of chemical compounds; 2) to determine the feasibility of pulsed electrical field for accelerating on-lees aging process; and, 3) to investigate the lexicon for the description of mouthfeel of Sauvignon blanc wine using napping®, and how these words relate to the chemical composition of wines.

7.1 Effect of different lees management techniques on the release of chemical compounds

In the current study, time and lees materials had a significant impact on the concentrations of total protein, primary amino acids nitrogen (PAN), neutral and acidic polysaccharides, mannoprotein, β-glucan and glycerol in model wines ($P < 0.001$). In addition, Inactivated yeast was a good source of amino acids, especially for Pro, Arg, Glu and Gln. Inactivated yeasts was a better source of polysaccharides than rehydrated yeasts and collected lees. The majority of the released polysaccharide was in the neutral form that reached a concentration ranging from 600 to 800 mg galactose/L on day 160 in model wine.

Both addition of β-glucanases and stirring frequency were minor factors that affected the release of chemical compounds from lees material. The current study demonstrated that addition of β-glucanases induced yeast autolysis and increased measured concentrations of protein, and neutral

polysaccharides and mannoprotein into model wine, but the effects were dependent on the lees type. Stirring frequency had a significant impact on the concentrations of protein, PAN and neutral polysaccharide ($P < 0.05$). Stirring frequency showed a statistical impact on protein, PAN and polysaccharide in model wines, but differences were small compared with the overall changes that were achieved at the end of the aging experiment.

The model wine system used in the current study is a useful method to demonstrate what happens in more compositional complex environments such as wine. Time and lees material were the major factors that affected the total protein, total free amino acids, polysaccharide, mannoprotein, and β-glucan in white winemaking (Chardonnay and sparkling wine) (Nunez et al., 2005; Suárez et al., 2005; and Torresi et al., 2014; Martínez-Lapuente et al., 2013; Juega et al., 2015).

In the current study, the model wine containing collected lees released the least chemical compounds during on-lees aging. As discussed earlier in Chapter 4, this was attributed to the removal of soluble materials during the pre-wash step prior to the addition to the model wine. As both collected lees and rehydrated yeasts were *Saccharomyces bayanus*, the model wine containing rehydrated yeasts was considered as representative of much commercial winemaking. In the model wine containing rehydrated yeasts, protein and total free amino acid changes during on-lees aging were consistent with the finding in sparkling wine (Nunez et al., 2005; Suárez et al., 2005). For example, the protein concentration increased initially, followed by a decrease and almost reached a plateau at the end of on-lees aging; total free amino acids increased throughout aging, and then became stable toward to the end of aging. The amounts of and trends in neutral polysaccharide concentration in both model and commercial wines were almost identical to those for total polysaccharide concentration due to the limited acidic polysaccharide concentration in wine. The neutral polysaccharide concentration in the model wine containing collected lees was about 100 mg galactose/L, which is similar to the result (80 mg/L, after 6 months on lees aging in Verdejo white wine) reported by Del Barrio-Galán et al. (2011). The difference might be attributed to the fact that 3% w/v fines lees was used in the previous study rather 5% w/v in the current study. The published information regarding the concentration of mannoprotein and β-glucan in the model wine containing rehydrated yeast and collected lees after 160 days of aging was limited.

The current study demonstrated that inactivated yeasts was a better source than rehydrated yeasts and collected lees for the release of polysaccharides and mannoproteins within a short aging period. Similarly, Wang (2014) compared the total polysaccharide concentration in Chardonnay wines exposed to ten different inactivated yeasts for four weeks, and demonstrated that all the wines containing inactivated yeasts had a significantly higher total polysaccharide concentration (range

200 to 300 mg glucose/L) than control (no exposure to inactivated yeasts, 150 mg glucose/L). In addition, Del Barrio-Galán et al. (2011) determined neutral polysaccharide concentration in Verdejo white wine after 6 months on lees aging, and the wine containing two yeast derivatives also had higher neutral polysaccharide concentration (100 mg/L) than the ones containing lees (70 mg/L).

The current study provided new information about application of β-glucanase and stirring frequency during aging in the presence of different lees material. Previously, Torresi et al. (2014) demonstrated during 12-month aging of a sparkling wine, that the addition of β-glucanases was not able to modify either the progression of yeast autolysis or the total protein concentration of the wine. Wang (2014) demonstrated an increase of polysaccharide concentration (from 200 mg/L to 600 mg/L) during 180 days on-lees aging with weekly stirring (stirring intensity and time were not specified). However, there was no control sample for the non-stirring treatment. The interaction between addition of enzyme, stirring frequency, aging time and lees material were not demonstrated in these studies. In the current study, throughout an aging period of 160 days, although stirring frequency and its interaction with time and lees material was statistical significant for neutral polysaccharide concentration in all model wines, the magnitude of this effect was small compared with the interaction between time, enzyme addition and lees material.

7.2 Feasibility of pulsed electric field for accelerating on-lees aging.

The consistency of the results between two aging experiments

Both rehydrated and inactivated yeasts were used in both on-lees aging experiments (Chapters 4 and 5). Periodic stirring (stirred every 4 days) and enzyme addition (0 and 7.5U of β-glucanases) were also common treatments. The results and trends in the concentrations of nitrogenous compounds, polysaccharides, mannoprotein, β-glucan and glycerol were very consistent between the two experiments. For example, the total free amino acids and mannoprotein concentration in both experiments were increased during on-lees aging. For example, the concentration of total free amino acids at the beginning and end of the second experiment (chapter 5) were 450 and 1200 µmol/L, respectively. These were only slightly lower than the results found in the first experiment (500 and 1500 µmol/L) (Chapter 4). This small difference can be attributed to the pre-wash step prior to PEF treatment in the second experiment, because the pre-wash step removes water soluble compounds (e.g. soluble proteins and amino acids) from yeast cells. However, polysaccharides (e.g. mannoprotein, 20 – 30 mg/L; and β-glucan, 4 -6 mg/L) remain attached to the yeast cells and were not affected by the pre-wash step. However, minor differences were observed in the concentration of total protein, PAN, total free amino acids and glycerol in the model wines containing rehydrated yeasts. Therefore, the effects of conventional lees management on releasing chemical compounds

during on-lees aging can be compared with those of the ones treated by the pulsed electric field.

Effects of PEF treatments

The current study has shown it is feasible to accelerate on-lees aging with pulsed electric field. The experiment demonstrated that the application of PEF (5.5 and 10.0 kV/cm field strength) to lees (5% w/v, rehydrated yeasts) provoked a release of intracellular materials, such as nitrogenous compounds, polysaccharides, and mannoprotein. In addition, time and lees materials also had a significant impact on the concentrations of total protein, primary amino acids nitrogen (PAN), neutral and acidic polysaccharides, mannoprotein, β-glucan and glycerol in model wines ($P < 0.001$).

PEF induced rehydrated yeasts to release three times more protein than the control at the beginning of the aging experiment. This finding is in accordance with a previous study by Ganeva et al. (2001) who applied a PEF treatment (4 to 4.5 kV/cm) to fresh *Kluyveromyces lactis* cells suspended in deionized water, and found that up to 50% of the total protein was released into the medium (phosphate buffered saline)within 24 hours. However, the actual concentration of the total protein in the medium was not stated. The release of peptides and/or free amino acids were significantly increased by PEF treatment. At the beginning of the aging experiment, higher concentration of PAN and total free amino acids were found in PEF treated samples than control, but these differences were small compared to the overall results. Opposite results were reported by Shynkaryk et al. (2009) in that PEF accelerated the release of the low molecular weight components but was not effective for high molecular weight intracellular compounds. Shynkaryk et al. (2009) applied PEF treatment (10 kV/cm) to a suspension of wine yeast cells (*Saccharomyces cerevisiae bayanus*, strain DV10), and determined protein released using SDS-PAGE; the results demonstrated that intracellular compounds with molecular weight between 32.5 to 78 kDa were released. As discussed in Chapter 4, the type of lees material had a major impact on the chemical composition of the released compounds during on-lees aging. Therefore, such opposing findings might be attributed to the different yeast strains that were used in these experiments. However, differential release of the yeast intracellular content could be an interesting strategy to manipulate wine composition and its sensory attributes. This should be considered for future work.

Previous studies mainly focused on the effect of PEF on the release of proteins and enzymes from different yeasts (Ganeva et al., 2001; Ganeva et al., 2003; Liu et al., 2013). Less attention was paid to polysaccharides, so the current study provided new information on the application of PEF to the release of polysaccharides. PEF released approximately 70 to 100 mg galactose/L more neutral polysaccharides to the model wine at the beginning of the aging experiment compared to the control. In addition, the higher PEF intensity caused greater damage to yeast cell walls, which led to

greater release of polysaccharides to the model wine. No mannoprotein was detected in both PEF treated and control samples on Day 0. Thus, although the yeast cell had lost its integrity, mannoproteins were still attached to the cell. Similar to the findings of Martínez et al. (2016), a higher amount of mannoprotein was released from PEF treated *S. cerevisiae* cells compared to the untreated sample on Day 1. On Day 32, higher mannoprotein concentration was found in the PEF treated samples than the control. However, no differences in mannoprotein concentration between all samples were found on Day 56. This indicated that PEF treatment was able to accelerate the release of mannoprotein from the yeast cell wall.

Even though PEF treatment on rehydrated yeasts released more protein and polysaccharides than the untreated ones, inactivated yeast was still the best source of these compounds because of its instaneous release of large amounts of neutral polysaccharide and mannoprotein in the model wine. For example, 850 mg galactose/L of neutral polysaccharide and 100 mg/L of mannoprotein were released in the model wine once inactivated yeasts were suspended in the model wine; the release of the mannoprotein can be further improved by using β-glucanases (7.5U). Inactivated yeasts also released much greater amounts of free amino acids than rehydrated yeasts. Therefore, contact with inactivated yeasts in the presence of β-glucanases would be the best choice for of the addition of polysaccharides and mannoprotein to wine.

Cost of using inactivated yeasts and PEF treatment

The retail price of Optilees is NZ$65/kg in New Zealand. Based on the dosage of inactivated yeasts used in the current study (5% w/v), 1 kg of Optilees is enough for making approximately 200 litres of white wine; thus the cost of aging wine on inactivated yeasts is about 33 cents per litre. However, the legal dosage of adding yeast derivatives to wine is lower in some wine producing countries. For example, the use of yeast cell wall preparations shall not be greater than 40 g/hL over all winemaking stages (EC Regulation 606/2009). Therefore, the cost per bottle could be as low as 3 cents. However, in this study, the dosage of inactivated lees was much higher than this (200 g/hL), but was chosen to be equivalent to that commonly used with collected lees. The effect of the dosage rate of inactivated yeasts on the amount of the chemical compounds released should be investigated in future work to confirm its usefulness.

In comparison, the capital cost of the pilot PEF system used in the current study is about NZ$500,000. Sampedro et al. (2014) estimated the capital cost of a commercial scale PEF system (equipped with monopolar, square wave pulses, three pairs of chambers with heat recovery) used for orange juice pasteurization to be about NZ$3.15 million; and the overall cost (including cooling water consumption and total electricity consumption) for pasteurizing 1 litre of orange juice was

found to be about 6 cents. However, the PEF treatment for yeast induction per litre of wine should be less than orange juice. First, the current study demonstrated that field strengths of 5.5 and 10 kV/cm was efficient for inducing yeast autolysis, and these field strengths were much lower than the ones used for microbial inactivation (35 to 60 kV/cm) for orange juice; thus lower energy costs are expected. Second, the amount of fine lees need to be treated is only up to 5% w/v; thus less cost per litre of wine than orange juice. However, the effect of PEF treatment on the induction of yeast autolysis was still at pilot-scale in the current study. Further studies are required to determine results for: 1) the PEF treatment in continuous flow mode; 2) the PEF treatment in continuous flow mode with real wine lees.

7.3 Relationship between model wine and commercial wine chemical composition

Comparing the compositional data for model wines in aging experiments and the commercial wines revealed that most of the model wines had lower concentrations than the commercial wines. This was likely because model wines had a much simpler chemical composition than the commercial ones. However, two exceptions were found which were total protein and mannoprotein concentrations. Total protein concentration from the model wines ranged between 5 to 20 mg BSA/L; these were similar to commercial wines. The higher total free amino acids concentration found in commercial wines (3700 to 7800 µmol/L) compared to model wines (200 to 1600 µmol/L) can probably be attributed to the hydrolysis of proteins during on-lees aging. The model wines containing inactivated yeasts had a similar mannoprotein concentration (140 to 160 mg/L) as the selected on-lees aged wines (70 to 150 mg/L). This clearly shows the effectiveness of using inactivated yeasts for the addition of mannoprotein to white wines.

7.4 Lexicon and napping®

Perceived sweetness and acidity seemed to be the major criteria for sorting the commercial examples of *Sauvignon blanc* wines. In total, 253 descriptors were collected but more than half of these descriptors were used no more than twice on different occasions, and combination of synonyms was not able to reduce this number significantly. Descriptors that had a frequency of more than 20 or so were too few to make a comprehensive lexicon.

Even though no lexicon was created to describe the mouthfeel of Sauvignon blanc wines, three innovative ideas were developed and applied in the current study. First, selected LEGO® pieces (in Chapter 6) were used to train panellists who had no napping experience for the first time. After training, all panellists had a good understanding of procedures and common problems in napping®.

'LEGO® training' is easy to set up and very cost effective in that pieces can be re-used in different napping studies. Second, PPI was not able to demonstrate the interference of duplicate control samples on the position of the other wine samples on the consensus map if the coordinates of the duplicated samples were swapped. In the current study, the coordinates of control samples were reordered to generate new consensus maps: the variances of the new consensus maps were found consistent with the one obtained from the old consensus map; also, the reordering of the coordinates of control samples had no effects on RV coefficients. Furthermore, a final consensus map is normally generated after excluding panellists with RV coefficients less than 0.500. However, RV coefficients are not always helpful for generating final consensus map if all of the RV coefficients of the panellists are similar. The alternative method (described in Chapter 6) also provided a systematic way to create the final consensus map that overcame this problem. Third, a system for sorting descriptors was developed. This system was found not suitable for the current study because too many different descriptors were provided by 14 panellists. However, this system will still be very useful for characterizing of aromatic profiles of wines which already have a well-developed lexicon.

7.5 Relationship between chemical composition and sensory

There was no precise correlation between chemical composition and collected descriptors (especially for the ones with low frequency). In addition, there was no correlation between the distribution of the wines on the consensus map and their chemical compositions. For example, SPY1 and MR were both associated with the vectors for total protein, mannoprotein, β-glucan and polysaccharide concentration (Figure 6.2) in terms of chemical composition, but were perceived as the most different wines in napping (Figure 6.7). This was attributed to the complexity of wine composition and its interaction between different perceptions. In fact, the distribution of wines on the consensus map seemed to be associated with the vinification technique, namely, on-lees aging and vessels used during fermentation and post fermentation. Panellists were able to distinguish the standard and on-lees aged Sauvignon blanc wines (Figure 6.7).

In wine, mouthfeel involves the perceptions of astringency, temperature, prickling, body (weight, viscous body), and burning. In general, the perceptions of astringency, prickling and burning are associating with phenolic compounds, carbon dioxide and ethanol, respectively. For a long time, the chemical compounds associated with wine body remained unclear (Jackson, 2002). In more recent studies, body of white wine was found to have a significant correlation with viscosity, lactate, osmotic potential, magnesium and total extract (i.e. the difference between the specific gravity of wine and a corresponding aqueous ethanol solution), with viscosity being the most highly correlated with perceived viscous mouthfeel (Runnebaum et al., 2011). In the current study, the concentration

of alcohol, acids and phenolics were fixed in the model wine system. Therefore, the composition of polysaccharides and nitrogenous compounds were the major focus of the discussion of how the lees manipulation can potentially affect the mouthfeel of white wine.

The lees manipulations applied in the current study could potentially increase perceived viscosity/wine body of white wines due to the release of polysaccharide and nitrogenous compounds. Gawel et al. (2016) extracted polysaccharides from Chardonnay wine after 4 months of on lees aging. The extracted polysaccharides (150 mg/L) were then added to a white wine with a high level of phenolic substances. It found that the addition of 150 mg/L of extracted polysaccharide was sufficient to improve the perceived viscosity, especially in the wine containing a higher concentration of phenolic substances. Therefore, the application of inactivated, and rehydrated yeasts (with or without PEF treatment) seemed to be the most promising lees material for improving the perceived viscosity or wine body because 150 to 800 mg/L of neutral polysaccharides were released during the aging experiment. In addition, Gawel et al. (2016) also found that polysaccharides with the molecular weight between 13 to 93 kDa (a mixture of smaller molecular weight mannoprotein and arabinogalactan proteins) caused a significant increase in perceived viscosity at pH 3.6. This clearly showed that perceived viscosity of white wine also contingent upon on molecular weight of polysaccharide and wine pH. The molecular weight of the released polysaccharides in the current study was not determined, but the molecular weight of the major polysaccharide in the model wine containing rehydrated yeasts might be in a similar range to the one reported by Del Barrio-Galán et al. (2015). Del Barrio-Galán et al. (2015) demonstrated in red wine elaborated from the same yeast strain as the current study (*Saccharomyces cerevisiae*, EC1118) that polysaccharides with a molecular weight between 10 to 80 kDa were the most abundant. However, the molecular weight of the polysaccharides in the model wine with the addition of β-glucanases was not determined. Thus, further study on the molecular weight of the released polysaccharide from wine with added β-glucanases and their relationship with perceived viscosity needs to be investigated. Furthermore, Skogerson et al. (2009) demonstrated that a correlation between the concentration of amino acids and white wines with low, medium and high body; and that proline concentration in wine was found to be strongly correlated to perceived viscosity, with serine, glutamate, alanine and arginine also moderately correlated. In the current study, inactivated, rehydrated and PEF treated yeasts were the good source of the above amino acids.

The viscosity of the model wines was measured to investigate the effects of the lees manipulation on wine viscosities. The viscosity of the model wine (10% v/v aqueous ethanol) was 1.25 mPa.s. After lees manipulations, the viscosity of the model wine was increased, up to 1.705 mPa.s. This clearly

demonstrated that lee manipulation was able to improve wine viscosity. However, no significant differences ($P > 0.05$) in viscosity were foundbetween different lees manipulation treatments in the current study.

7.6 Specific findings related to best practice

The inactivated yeast (Optilees) used in the current study released more free amino acids, polysaccharides, mannoprotein and β-glucan than the other two lees materials (collected lees, rehydrate yeasts with/without PEF treatment). In a 56 days on-lees aging, its application with β-glucanase addition (7.5U, β-glucanase) and stirring every 4 days for 10 mins achieved the highest concentration of mannoprotein concentration among all the treatments. Attention needs to be paid when using inactivated yeast or other yeast products. The chemical composition and/or purity of different inactivated yeast or yeasts derivative products may vary. For example, Wang (2014) found that Chardonnay wine subjected to Mannostab had a higher total polysaccharide concentration (350 mg glucose/L) than Biolees (200 mg glucose/L). In addition, Pérez-Magariño et al. (2015) characterized four different inactivated yeast products based on purity of polysaccharide, mono- and polysaccharide composition; the purity, mannose and glucose percentages were ranged from 63 to 89%, 53 to 86%, and 14 to 47%, respectively. Thus, the amount of polysaccharides, mannoprotein and β-glucan release is dependent on the types of yeast product. In addition, the legal permitted dosage for yeast derivatives to wine is lower in some wine producing countries. For example, in the EU the use of yeast cell wall preparations shall not be over the limit of 40 g/hL over all different winemaking stages to give wine positive characteristics (EC Regulation 606/2009).

Rehydrated yeasts with pulsed electric field treatment (PL, rehydrated yeasts treated by low level PEF strength, 5.5 kV/cm, 16 μs, 5 Hz), an addition of 7.5U of β-glucanase, stirring frequency of every 4 days for 10 mins, and an aging time of 56 days might be an alternative choice. A shorter aging time (32 days) may be considered if increasing of mannoprotein concentration was the main purpose of the aging. However, the above operational parameters were based on a pilot-scale study and its application in product-scale is still required to be investigated in the further study. In addition, the capital and running cost of a PEF system needs to be considered.

Finally, the use of rehydrated yeasts with addition of 7.5U of β-glucanase, stirring frequency of every 4 days for 10 mins, and an aging time for 56 days can be an option. Although this choice released the least polysaccharides, this method most closely mirrors the situation in current commercial wine production. In addition, an aging time can be extended to up to 160 days to maximise the release of neutral polysaccharide from yeast cells.

7.7 Recommendations for future studies

In this study, the quantification and characterization of the compounds released from the collected lees, inactivated yeasts, rehydrated yeasts and PEF induced yeasts with/without periodic stirring and β-glucanase, together with the sensorial and chemical characterization of the selected commercial examples of New Zealand Sauvignon blanc wines had been carried out. Some aspects derived from this study might be investigated in future studies. These include:

- Further investigation of the release of nitrogenous compounds, polysaccharides, and mannoprotein from the non-*Saccharomyces cerevisiae* species during on-lees aging in model wine in order to compare with the present work.
- Further investigation the release of nitrogenous compounds, polysaccharides, and mannoprotein from the induced lees (both *Saccharomyces* and non-*Saccharomyces* species) in real wine aging.
- To specifically identify the polysaccharides released during on lees aging, and monitor the changes of their molecular weights; and try to link the types of polysaccharides and their molecular weights to wine mouthfeel.
- Further isolation and identification of mannoprotein by using 2-D gel.
- Further method development for mannoprotein isolation and spectrophotometric quantification.
- Improved napping methods for sensorial characterization of wine. This can be approached from the combination of descriptive analysis and polarised projective mapping.
- Polarized projective mapping (PPM) may be used for the future study to establish the lexicon for describing white wine mouthfeel. This method was first introduced by Teillet et al. (2010) in a sensory methodology study conducted with water tasting. More recently, Ares et al. (2013) continued the methodology study with orange-flavoured drinks. The principle behind this method is to sort samples (same as ordinary napping) against one or more poles; the poles are the selected samples which had gone through a sensorial and chemical characterization in the preliminary study (Ares et al., 2013). Therefore, the 'actual meaning' of the descriptive terms (especially those for qualitative description) can be checked against 'poles', thus, a combination of the descriptors can be done even though the perceptive threshold of panellists are different. However, this method is a novel napping® method, and further refining of the methodology for wine tasting needs to be investigated.

References

Adams, D. J. (2004). Fungal cell wall chitinases and glucanses. *Microbiology*, *150*, 2029-2035.

Akın, E., and G. A. Evrendilek. (2009). Effect of Pulsed Electric Fields on Physical, Chemical, and Microbiological Properties of Formulated Carrot Juice." *Food Science and Technology International*, *15*, 275–82.

Albert, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2002). *The Molecular Biology of the Cell*. New York: garland Science.

Allen, M. S., Lacey, M. J., Harris, R. L. N., Vance Brown, W. (1991). Contribution of methoxypyrazines to Sauvignon blanc wine aroma. *American Journal of Enology and Viticulture*, *42*, 109-112.

Alexandre, H., and Guilloux-Benatier, M. (2006). Yeast autolysis in Sparkling wine – a review. *Australian Journal of Grape & Research*, *12*, 119-127.

Alexandre, H., Heintz, D., Chassagne, D., Guilloux-Benatier, M., Charpentier, C., and Feuillat, M. (2001). Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. *Journal of Industrial Microbiology and Biotechnology*, *26*, 235-240.

Álvarez, Ignacio, Santiago Condón, and Javier Raso. (2006). Microbial Inactivation by Pulsed Electric Fields. In *Pulsed Electric Fields Technology for the Food Industry*, edited by Javier Raso and Volker Heinz, 97–129. Food Engineering Series. Springer US.

Ares, G., Barreiro, C., Deliza, R., Giménez, A., and Gámbaro, A. (2010). Application of a check-all-that-apply question to the development of chocolate milk desserts. *Journal of Sensory Studies*, *25*, 67-86.

Aslankoohi, E., Rezaei, M. N., Vervoort, Y., Courtin, C. M., Verstrepen, K. J. (2015). Glycerol production by fermenting yeast cells is essential for optimal bread dough fermentation. *PLOS ONE*. *Doi:10.1371/journal.pone.0119364*.

Bakker, J., and Clarke, R. J. (2nd Ed)(2011). Wine: Flavour Chemistry. Oxford, UK: Blackwell Publishing Ltd.

Bautista, R., Fernández, E., and Falqué, E. (2007). Effect of the contact with fermentation-lees or commercial-lees on the volatile composition of white wines. *European Food Research & Technology*, *224*, 405-413.

Benítez, T., Rincón, A. M., & Codón, A. C. (2011). Chapter 3 - Yeasts Used in Biologically Aged Wines.

In V. C. S. Alfonso, M. Rosario, R. M. Ramon Gonzalez GarciaA2 - Alfonso V. Carrascosa Santiago & G. Ramon Gonzalez (Eds.), *Molecular Wine Microbiology* (pp. 51-84). San Diego: Academic Press.

Bisson, L. F., Joseph, C. M. L. (2009). Yeasts. In H. König, Unden, G., Fröhlich, J. (Ed.), *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 47-60). Berlin: Springer.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248-254.

Brennan, J. G. (2006) (ed). *Food Processing Handbook*. Weinheim, Germany: Wiley-VCH.

Brianceau, Sylène, Mohammad Turk, Xavier Vitrac, and Eugène Vorobiev (2014). Combined Densification and Pulsed Electric Field Treatment for Selective Polyphenols Recovery from Fermented Grape Pomace. *Innovative Food Science & Emerging Technologies*, doi:10.1016/j.ifset.2014.07.010.

Brissonnet, F., and Maujean, A. (1991). Identification of some foam-active compounds in a Champagne base wine. *American Journal of Enology and Viticulture*, *44*, 97-102.

Boivin, S., Feuillat, M., Alexandre, H., Charpentier, C. (1998). Effect of must turbidity on cell wall porosity and macromolecule excretion of Saccharomyces cerevisiae cultivated on grape juice. American Journal of Viticulture and Enology¸49, 325-332.

Bozdoğan, A., and Canbaş, A. (2012). The effect of yeast strain, immobilisation, and aging time on the amount of free amino acids and amino acids in peptides of Sparkling wines obtained from cv. Dimrit grapes. *South African Journal of Enology and Viticulture*, *33*, 257-263.

Bueno, J. E., Peinado, R. A., Medina, M., and Moreno, J. (2006). Effect of a short contact time with lees on volatile composition of Airen and Macabeo wines. *Biotechnology Letter*, *28*, 1007-1011.

Bustamante, M. A., Moral, R., Paredes, C., Pérez-Espinosa, A., Moreno-Caselles, J., & Pérez-Murcia, M. D. (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. *Waste Management, 28*(2), 372-380.

Buxaderas, S., and López-Tamames, E. (2012). Sparkling wines: features and trends from tradition. *Advances in Food & Nutrition Research*, *66*, 1-45.

Canal-Llaubères, R. M. (2010). 4 - Enzymes and wine quality. In A. G. Reynolds (Ed.), *Managing Wine Quality* (pp. 93-132): Woodhead Publishing.

Calderone G., Naulet, N., Guillou, C., and Reniero, F. (2004). Characterization of European wine glycerol: stable carbon isotope approach. *Journal of Agricultural and Food Chemistry*, *52*, 5902-5906.

Caridi, A. (2006). Enological functions of parietal yeast mannoproteins. *Antonie van Leeuwenhoek*, *89*, 417-422.

Castro, Armando J., Gustavo V. Barbosa-Cánovas, and Barry G. Swanson (1993). Microbial inactivation of foods by pulsed electric fields. *Journal of Food Processing and Preservation, 17*, 47–73.

Cebollero, E., Carrascosa, A. V., and Gonzalez, R. (2005). Evidence for yeast autophagy during simulation of Sparkling wine aging: A reappraisal of the mechanism of yeast in wine. *Biotechnology Progress*, *21*, 614-616.

Charpentier, C. (2010). Ageing on lees (sur lies) and the use of speciality inactive yeasts during wine fermentation. In A. G. Reynolds (Ed.), *Managing wine quality* (Vol. 2, pp. 164-187). Oxford: Woodhead Publishing.

Charpentier, C., Assenac, J., Charpentier, M., Prome, J. C., Duteurtre, B., and Feuillat, M. (2005). Release of nucleotides and nucleosides during yeast autolysis: kinetics and potential impact on flavour. *Journal of Agricultural & Food Chemistry, 53*, 3000-3007.

Charpentier, C., Dos Santos, A. M., and Feuilaat, M. (2004). Release of macromolecules by *Saccharomyces cerevisiae* during aging on French flor sherry wine 'Vin jaune'. *International Journal of Food Microbiology*, *96*, 253-262.

Charpentier, C., Feuillat, M. (1993). Yeast autolysis. In *Wine microbiology and biotechnology.* (Ed, pp. 225–242). Chur, Switzerland: Harwood Academic Publishers.

Charpentier, C., & Feuillat, M. (2008). Elevage des vins rouges sur lies.Incidence d l'addition d'une β-glucanase sur lacomposition en polysaccharides et leurs interaction avec polyphenols. *Rev. Enologues, 129*, 31-35.

Chen, Y. Q., and Klionsky, D. J. (2011). The regulation of autophagy – unanswered questions. *Journal of Cell Science*, *124*, 161-170.

Comuzzo, P., Tat, L., Tonizzo, A., and Battistutta, F. (2006). Yeast derivatives (extracts and autolysates) in winemaking: release of volatile compounds and effects on wine aroma volatility. *Food Chemistry*, *99*, 217-230.

Corrales, M., Toepfl, S., Butz, P., and Knorr, D. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: a comparison. *Innovative Food Science and Emerging Technologies*, *9*, 85-91.

Courtis, K., Todd, B., and Zhao, J. (1998). The potential role of nucleotides in wine flavour. *Australian Grapegrower and winemaker*, *409*, 51-53.Couto, J. A., Filipe, N., Francisco, C., and Tim, H. (2005). Thermal Inactivation of the Wine Spoilage Yeasts Dekkera/Brettanomyces. *International Journal of Food Microbiology, 104*, 337–44.

Dang, C. (2013). Physico-chemical properties and mouthfeel in New Zealand Pinot Noir wines. Lincoln University, New Zealand.

Darra, N. El., Nabil, G., Eugène, V., Maroun, R. G., and Louka, N. (2013). Pulsed Electric Field-Assisted Cold Maceration of Cabernet Franc and Cabernet Sauvignon Grapes. *American Journal of Enology and Viticulture, 64*, 476–84.

Del Barrio-Galán, R., Pérez-Magariño, S., Ortega-Heras, M., Williams, P., and Doco, T. (2011). Effect of aging on lees and of three different dry yeast derivative products on Verdejo white wine composition and sensorial characteristics. *Journal of Agricultural & Food Chemistry*, *59*, 12433-12442.

Del Barrio-Galán, R., Pérez-Magariño, S., Ortega-Heras, M., Guadalupe, Z., and Ayestarán, B. (2012). Polysaccharide characterization of commercial dry yeast preparations and their effect on white and red wine composition. *LWT-Food Science and Technology*, *48*, 215-223.

Delsart, C., Ghidossi, R., Poupot, C., Cholet, C., Grimi, N., Vorobiev, E., Milisic, V., and Mietton-Peuchot, M. (2012). Enhanced extraction of phenolic compounds from Merlot grapes by pulsed electric field treatment. *American Journal of Enology and Viticulture*, *63*, 205-211.

Delsart, C., Cholet, C., Ghidossi, R., Grimi, N., Gontier, E., Gény, L., Vorobiev, E., and Mietton-Peuchot, M. (2014). Effects of pulsed electric fields on Cabernet sauvignon grape berries and on the characteristics of wines. *Food and Bioprocess Technology*, *7*, 424-436.

Doco, T., Vuchot, P., Cheynier, V., and Moutounet, M. (2003). Structural modification of wine arabinogalactans during aging on lees. *American Journal Enology and Viticulture*, *54*, 150-157.

Donsì, F., Ferrari, G., Fruilo, M., and Pataro, G. (2011). Pulsed electric fields – assisted vinification. *Procedia Food Science, 11th International Congress on Engineering and Food* (ICEF11) *1*, 780-785.

Douwes, J. (2005). (1→3)-β-D-glucans and respiratory health: a review of the scientific evidence. *Indoor Air, 15*, 160-169.

Dupin, I. V. S., McKinnon, B. M., Ryan, C., Boulay, M., Markides, A. J., Jones, G. P., Williams, P. J., and Waters, E. J. (2000). *Saccharomyces cerevisiae* Mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *Journal of Agriculture and Food Chemistry*, *48*, 3098-3105.

Escot, S., Feuillat, M., Dulau, L., and Charpentier, C. (2001). Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Australian Journal of Grape & Wine Research, 7*, 153-159.

Elez-Martínez, Pedro, Ángel Sobrino-López, Robert Soliva-Fortuny, and Olga Martín-Belloso. "Chapter 4 - Pulsed Electric Field Processing of Fluid Foods." In *Novel Thermal and Non-Thermal Technologies for Fluid Foods*, edited by P. J. Cullen, Brijesh K. Tiwari, and Vasilis P. Valdramidis, 63–108. San Diego: Academic Press, 2012.

Evrendilek, G. Akdemir, Z. T Jin, K. T Ruhlman, X Qiu, Q. H Zhang, and E. R Richter. (2000). Microbial Safety and Shelf-Life of Apple Juice and Cider Processed by Bench and Pilot Scale PEF Systems. *Innovative Food Science & Emerging Technologies, 1*, 77–86.

Feldmann, H. (2012). *Yeast: molecular and cell biology*. Weinheim: Wiley-Blackwell Publishing.

Feuillat, M. (2003). Yeast Macromolecules: Origin, Composition, and Enological Interest. *American Journal of Enology and Victiculture, 54*, 211-213.

Feuillat, M., & Charpentier, C. (1982). Autolysis of Yeasts in Champagne. *American Journal of Enology and Viticulture*, *33*(1), 6–13.

Fernández, O., Martínez, O., Hernández, Z., Guadalupe, Z., and Ayestarán, B. (2011). Effect of the presence of lysated lees on polysaccharides, colour and main phenolic compounds of red wine during barrel aging. *Food Research Internation*, *44*, 84-91.

Flanzy, C., Salgues, M., Bidan, P., Dubois, C., Moulin, J. P., and Sablayrolles, J. M. (1999). In Oenology: Fondements scientifiques et technologiques (Flanzy, C. ed.), 497-516. Technique et Documantation, Paris, France.

Fornairon-Bonnefond, C., Camarasa, C., Moutounet, M., & Salmon, J. M. (2002). New trends on yeast autolysis and wine ageing on lees: a bibliographic review. *Journal of International des Science de la Vigne et du Vin, 36*, 49-69.

Frevert, J., and Ballou, C. E. (1985). *Saccharomyces cerevisiae* structural cell wall mannoprotein. *Biochemistry, 24*, 753-759.

Gallart, M., López-Tamames, Suberbiola, G., and Buxaderas, S. (2002). Influence of fatty acids on wine foaming. *Journal of agricultural and Food Chemistry*, *50*, 7042-7045.

Ganeva, V., Galutzov, B., and Teissie, J. (2014). Evidence that pulsed electric field treatment enhances the cell wall porosity of yeast cells. *Applied Biochemistry and Biotechnology*, *173(3)*, 1540-1552.

Garrett, R. H., and Grisham, C. M. (2010) (ed.). Biochemistry. Brooks/Cole. USA.

Garde-Cerdán, T., L. González-Arenzana, N. López, R. López, P. Santamaría, and I. López-Alfaro. (2013). Effect of Different Pulsed Electric Field Treatments on the Volatile Composition of Graciano, Tempranillo and Grenache Grape Varieties. *Innovative Food Science & Emerging Technologies, 20*, 91–99.

Garde-Cerdán, T. L., Marsellés-Fontanet, A. R., Arias-Gil, M., Ancín-Azpilicueta, C., and Martín-Belloso, O. (2008). Influence of SO2 on the Evolution of Volatile Compounds through Alcoholic Fermentation of Must Stabilized by Pulsed Electric Fields. *European Food Research and Technology, 227*, 401–408.

Garde-Cerdán, T., Marsellés-Fontanet, R. A., Arias-Gil, M., Martín-Belloso, O., and Ancín-Azpilicueta, C. (2007). Influence of SO_2 on the Consumption of Nitrogen Compounds through Alcoholic Fermentation of Must Sterilized by Pulsed Electric Fields. *Food Chemistry, 103*, 771–777.

Giacalone, D., Ribeiro, L. M., and Frøst, M. B. (2013). Consumer-based product profiling: application of partial Napping® for sensory characterization of specialty beers by novices and experts. *Journal of Food Products Marketing*, *19*, 201-218.

Gómez, M. E., Igartuburu, J. M., Pando, E., Luis, F. R., & Mourente, G. (2004). Lipid Composition of Lees from Sherry Wine. Journal of Agricultural and Food Chemistry, 52(15), 4791-4794. doi: 10.1021/jf030499r

Gonçalves, F., Heyraud, A., de Pinho, M. N., Rinaudo, M. (2002). Characterization of white wine

mannoproteins. *Journal of Agricultural and Food Chemistry*, *50*(21), 97-101.

González-Marco, A., and Ancin-Azpilicueta, C. (2006). Influence of lees contact on evolution of amines in Chardonnay wine. *Journal of Food Science*, *71*, 544-548.

Góngora-Nieto, M. M., Sepúlveda, D. R., Pedrow, P., Barbosa-Cánovas, G. V., and Swanson, B. G. (2002). Food Processing by Pulsed Electric Fields: Treatment Delivery, Inactivation Level, and Regulatory Aspects*. LWT - Food Science and Technology, 35*, 375–388.

Grimi, N., Lebovka, N. I., Vorobiev, E., and Vaxelaire, J. (2009). Effect of a pulsed electric field treatment on expression behaviour and juice quality of Chardonnay grape. *Food Biophysics*, *4*, 191-198.

Guadalpule, Z., Palacikos, A., and Ayestarán, B. (2007). Maceration enzymes and mannoproteins: a possible strategy to increase colloidal stability and colour extraction in red wines. *Journal of Agricultural and Food Chemistry*, *55*, 4854-4862.

Guadalupe, Z., and Ayestarán, B. (2007). Polysaccharide profile and content during the vinification and aging of Tempranillo red wines. *Journal of Agriculture and Food Chemistry*, *55*, 10720-10728.

Guadalupe, Z., and Ayestarán, B. (2007). Effect of commercial mannoprotein addition on polysaccharide, polyphenolic and colour composition in red wines. *Journal of Agriculture and Food Chemistry*, *56*, 9022-9029.

Gawl, R., Smith, P. A., and Waters, E. J. (2017) Influence of polysaccharides on the taste and mouthfeel of white wine. *Australian Journal of Grape and Wine Research*, *22*, 350-357.

Guilloux-Benatier, M., and Chassagne, D. (2003). Comparison of components released by fermented or active dried yeasts after aging on lees in a model wine. *Journal of Agricultural and Food Chemistry*, *51*, 746-751.

Hopfer, H., and Heymann, H. (2013). A summary of projective mapping observations – The effect of replicates and shape, and individual performance measurements. *Food Quality and Preference*, *28*, 164-181.

Humbert-Goffard, A., Saucier, C., Moine-Ledoux, V. M., Canal-Llaubères, R., Dubourdieu, D., and Glories, Y. (2004). An assay for glucanase activity in wine. *Enzyme and Microbial Technology*, *34*, 537-543.

Iland, P., Ewart, A., Sitters, J., Markides, A., and Bruer, N. (2000). Techniques for chemical analysis and quality monitoring during winemaking. Patrick Iland Wine Promotions.

Jackson, R. S. (2008). *Wine Science: Principles and Applications*. Academic Press.

Jackson, R. S. (2008). *Wine tasting*: *A Professional Handbook*. Academic Press.

Jiménez-Moreno, N., and Ancín-Azpilicueta, C. (2007). Binding of oak volatile compounds by wine lees during simulation of wine aging. *LWT-Food Science and Technology*, *40*, 619-624.

Jiménez-Moreno, N., and Ancín-Azpilicueta, C. (2009). Sorption of volatile phenols by yeast cell walls. *International Journal of Wine Research*, *1*, 11-18.

Juega, M., Carrascosa, A. V., and Martínez-Rodríguez, A. J. (2015). Effect of short aging on lees on the mannoprotein content, aromatic profile, and sensorial character of white wines. *Journal of Food Science*, *80*, 384-388.

Kennedy, J., and Heymann, H. (2009). Projective mapping and descriptive analysis of milk and dark chocolates. Journal of Sensory Studies, 24, 220-233.

Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988). Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, *170*, 2683-2686.

Klis, F. M., Mol, P., Hellingwerf, K., and Brul. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*, *26*, 239-256.

Klosse, P. (2013). Umami in wine. *Research in Hospitality Management*, *2*: 1-2, 25-58, DOI: 10.1080/22243534.2013.11828287.

Kollar, R., Reinhold, B. B., Petráková, E., Yeh, H. J. C., Ashwell, G., Drgonová, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997). Architecture of the yeast cell wall. *The Journal of Biological Chemistry*, *272*, 17762-17775.

Kocková-Kratochvílová, A. (1990). Yeasts and yeast-like organisms. New York, NY, USA: VCH.

Krainer, E., Stark, R. E., Naider, F. K., Alagramam, K., and Becker, J. M. (1994). Direct observation of cell wall glucans in whole cells of *Saccharomyces cerevisiae* by magic-angle spinning ^{13}C-NMR. *Biopolymers*, *34*, 1627-1635.

Leong, S. Y., Burritt, D. J., and Oey, I. (2016). Evaluation of the anthocyanin release and health-

promoting properties of Pinot Noir grape juices after pulsed electric fields. *Food Chemistry*, *196*, 833-841.

Leroy, M. J., Charpentier, M., Duteurtre, B., Feuillat, M., and Charpentier, C. (1990). Yeast autolysis during Champagne aging. *American Journal of Enology and Viticulture*, *41*, 21-28.

Liberatore, M. T., Pati, S., Del Nobile, M. A., and La Notte, E. (2010). Aroma quality improvement of chardonnay white wine by fermentation and aging in barrique on lees. *Food Research International*, *43*, 996-1002.

Lipke, P. N., and Ovalle, R. (1998). Cell wall architecture in Yeast: new structure and new challenges. *Journal of Bacteriology¸180*, 3735-3740.

Liu, D., Lebovka, N. I., and Vorobiev, E. (2013). Impact of electric pulse treatment on selective extraction of intracellular compounds from *Saccharomyces cerevisiae* yeasts. *Food and Bioprocess Technology*, 576-584.

Liu, X. J., Yin, Y. G., Fan, S. M., Zhu, C., and Luo, G. C. (2006). GC-MS analysis of aroma component variation of wine treated by high voltage pulsed electric field. *Food Science, 27*, 654-657.

Liu, J., Grønbeck, M. S., Di Monaco, R., Giacalone, D., Bredie, W. L. P. (2016). Performance of flash profile and Napping with and without training for describing small sensory differences in a model wine. *Food Quality and Perference*, *48*, 41-49.

Loira, I., Vejarano, R., Morata, A., Ricardo-da-Silva, J. M., Laureano, O., González, M. C., and Suárez-Lepe, J. A. (2013). Effectr of *Saccharomyces* strains on the quality of red wines aged on lees. *Food Chemistry*, *139*, 1044-1051.

Losada, M. M., López, J. F., Añón, A., Andrés, J., and Revilla, E. (2012). Influence of some oenological practices on the aromatic and sensorial characteristics of white Verdejo wines. *International Journal of Food Science & Technology*, *47*, 1826-1834.

López-Alfaro, I., González-Arenzana, L., López, N., Santamaría, P., López, R.,and Garde-Cerdán, T. (2013). Pulsed electric field treatment enhanced stilbene content in Graciano, Tempranillo and Grenache grape varieties. *Food Chemistry*, *141*, 3759-3765.

López, N., Puértolas, E., Condón, S., Álvarez, I., and Raso, J. (2008). Application of pulsed electric fields for improving the maceration process during vinification of red wine: influence of grape variety. *European Food Research and Technology, 227*, 1099–1107.

López, N., Puértolas, E., Hernández-Orte, P., Álvarez, I., and Raso, J. (2009). Effect of a pulsed electric field treatment on the anthocyanins composition and other quality parameters of Cabernet Sauvignon freshely fermented model wines obtained after different maceration times. *LWT – Food Science and Technology, 42*, 1225-1231.

Loira, I., Vejarano, R., Morata, A., Ricardo-da-Silva, J. M., Laureano, O., González, M. C., and Suárez-Lepe, J. A. (2013). Effect of *Saccharomyces* strains on the quality of red wines aged on lees. *Food Chemistry*, *139*, 1044-1051.

Louw, L., Malherbe, S., Naes, T., Lambrechts, M., van Rensbury, P., Nieuwoudt, H. (2013). Validation of two Napping® techniques as rapid sensory screening tools for high alcohol products. *Food Quality and Preference*, *30*, 192-201.

Lozano, J., Santos, J. P., and Horrillo, M. C. (2005). Classification of white wine aromas with an electric nose. *Talanta*, *67*, 610-616.

Lucero, P., Peñalver, E., Moreno, E., and Lagunas, R. (2000). Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *66*, 4456-4461.

Luo, Y., Wang, J., Liu, B., Wang, Z., Yuan, Y., and Yue, T. (2015). Effect of yeast cell morphology, cell wall physical structure and chemical composition on patulin adsorption. *PLOS ONE*, *10*, 1-16.

Marcel Van Der Vaart, J., Caro, L. H. P., Chapman, J. W., Klis, F. M., and Theio Verrips, C. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, *177*, 3104-3110.

Margalit, Y. (3rd Ed)(2012). *Concept in Wine Chemistry*. San Francisco: Board and Bench Publishing.

Marsellés-Fontanet, À., Robert, A. P., Paola, O., Mínguez-Sanz, S., and Martín-Belloso, O. (2009). Optimising the inactivation of grape juice spoilage organisms by pulse electric fields. *International Journal of Food Microbiology*, *130*, 159–65.

Martínez, J. M., Cebrián, G., Álvarez, I., and Raso, J. (2016). Release of Mannoproteins during *Saccharomyces cerevisiae* autolysis induced by pulsed electric field. *Frontiers in Microbiology*, *7*, 1435.

Martínez, J. M., Delso, C., Aguilar, D., Cebrián, G, Álvarez, I., and Raso, J. (2018). Factors influencing autolysis of *Saccharomyces cerevisiae* cells induced by pulsed electric fields. *Food Microbiology*, *73*,

67-72.

Martínez-Lapuente, L., Guadalupe, Z., Ayestarán, B., Ortega-Heras, M., and Pérez-Magariño, S. (2013). Sparkling wines produced from alternative varieties: sensory attributes and evolution of phenolics during winemaking and aging. *American Journal of Enology and Viticulture*, *64*, 39-49.

Martínez-Rodríguez, A. J., and Polo, M. C. (2000). Characterization of the Nitrogen Compounds Released during Yeast Autolysis in a Model Wine System. *Journal of Agricultural and Food Chemistry*, *48*(4), 1081–1085.

Martínez-Rodríguez, A. J., and Pueyo, E. (2009). Sparkling wines and yeast autolysis. Moreno-Arribas, M. V., and Polo, M. C. *Wine Chemistry and Biochemistry*, 61-75. Springer, New York.

Martínez-Rodríguez, A. J., Carrascosa, A. V., and Polo, M. C. (2001). Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *International Journal of Food Microbiology*, *68*, 155-160.

Masino, F., Montevecchi, G., Arfelli, G., and Antonelli, A. (2008). Evaluation of the combined effects of enzymatic treatment and aging on lees on the aroma of wine from *Bombino bianco* grapes. *Journal of Agricultural & Food Chemistry*, *56*, 9495-9501.

Masino, F., Montevecchi, G., Arfelli, G., and Antonelli, A. (2009). Evaluation of the combined effects of enzymatic treatment and aging on lees on the aroma of wine from Bombino bianco grapes. *Journal of Agricultural and Food Chemistry*, *56*, 9495-9501.

Matile, P., Cortat, M., Wiemken, A., and Frey-Wyssling, A. (1971). Isolation of Glucanse-containing particles from budding *Saccharomyces cerevisiae*, *68*, 636-640.

Mazauric, J. P., and Salmon, J. M. (2006). Interactions between yeast lees and wine polyphenols during simulation of wine aging: II. Analysis of desorbed polyphenol compounds from yeast lees. *Journal of Agricultural & Food Chemistry, 54*, 3876-3881.

McNamee, C., Noci, F., Cronin, D. A., Lyng, J. G., Morgan, D. J., and Scannell, A. G. M. (2010). PEF based hurdle strategy to control *Pichia fermentans*, *Listeria innocua* and *Escherichia coli k12* in Orange Juice. *International Journal of Food Microbiology, 138*, 13–18.

Medina, K., Boido, E., Dellacassa, E., and Carrau, F. (2005). Yeast interactions with anthocyanins

during red wine fermentation. *American Journal of Enology & Viticulture, 56*, 104-109.

Misako, K., Atsushi, O., and Yoichi, U. (2002). Taste enhancements between various amino acids and IMP. *Chemical Senses Journal*, *27*, 739-745.

Moine-Ledoux, V., Perrin, A., Paladin, I., and Dubourdieu, D. (1997). First results of tartaric stabilisation by adding mannoproteins. *Journal International des Sciences de la Vigne et du Vin*, *31*, 23-31.

Morata, A., Gómez-Cordvés, M. C., Suberviola, J., Bartolomé, B., Colomo, B., and Suárez, J. A. (2005). Adsorption of anthocyanins by yeast cell walls during the fermentation of red wine. *Journal Agricultural & Food Chemistry, 51*, 4084-4088.

Morata, A., Gómez-Cordvés, M. C., Colomo, B., and Suárez, J. A. (2005). Cell wall anthocyanin adsorption by different *Saccharomyces* strains during the fermentation of *Vitis Vinifera* L. cv Graciano grapes. *European Food Research & Technology*, *220*, 341-346.

Moreno-Arribas, M. V., Bartolomé, Pueyo, E., & Polo, M. C. (1998). Isolation and Characterization of Individual Peptides from Wine. *Journal of Agricultural and Food Chemistry*, *46*(9), 3422–3425.

Moreno-Arribas, V., Pueyo, E., Polo, C., and Martín-Álvarez, P. J. (1998). Changes in the amino acid composition of the different nitrogenous fractions during the aging of wine with yeasts. *Journal of Agricultural and Food Chemistry*, *46*, 4042-4051.

Moreno-Arribas, V., Pueyo, E., and Polo, C. (1996). Peptides in musts and wines. Changes during the manufacture of Cavas (Sparkling wines). *Journal of Agricultural and Food Chemistry*, *44*, 3783-3788.

Moreno-Arribas, M. V., Pueyo, E., Nieto, F. J., Martin-Alvarez, P. J., and Polo, M. C. (2000). Influence of the polysaccharides and nitrogen compounds on foaming properties of sparkling wines, *Food Chemstry*, *70*, 309-317.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., and Martín-Belloso, O. (2007). Influence of treatment time and pulse frequency on *Salmonella Enteritidis, Escherichia Coli* and *Listeria Monocytogenes* populations inoculated in melon and watermelon juices treated by pulsed electric fields. *International Journal of Food Microbiology, 117*, 192–200.

Nestrud, M. A., and Lawless, H. T. (2011). Recovery of subsampled dimensions and configurations derived from napping data by MFA and MDS. *Attention, Perception and Psychophysics*, *73*, 1266-1278.

Nieuwoudt, H. H., Prior, B. A., Pretorius, I. S., and Bauer, F. F. (2017). Glycerol in South African table wines: an assessment of its relationship to wine quality. *South African Journal of Enology and Viticulture*, *23*, 22-30.

Neto, F. S., de Castilhos, M, B., Telis, V. R., and Telis-Romero, J. (2014). Effect of ethanol, dry extract and reducing sugars on density and viscosity of Brazilian red wines. *Journal of Science of Food and Agriculture*, *95*, 1421-1427.

Oruna-Concha, M. J., Methven, L., Blumenthal, H., Young, C., and Mottram, D. S. (2007). Differences in glutamic acids and 5'-ribonucleotide contents between flesh and pulp of tomatoes and the relationship with Umami taste. *Journal of Agricultural and Food Chemistry*, *55*, 5776-5780.

Pagès, J. (2005).Collection and analysis of perceived product inter-distances using multiple factor analysis: application to the study of 10 white wines from the Loire Valley. *Food Quality and Preference*, *16*, 642-649.

Pagès, J. (2003). Direct collection of sensory distances: applicationto the evaluation of 10 white wines of the Loire Valley. *Sciences des Aliments*, *23*, 679-688.

Palacios, S., Vasserot, Y., and Maujean, A. (1997). Evidence for sulphur volatile products adsorption by yeast lees. *American Journal of Enology & Viticulture*, *48*, 525-526.

Palomero, F., Morata, A., Benito, S., González, Suárez-Lepe, J. A. (2007). Conventional and enzyme-assisted autolysis during aging over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chemistry*, *105*, 838-846.

Palomero, F., Bertani, P., Simón, B. F., Cadahía, E., Benito, S., Morta, A., and Suárez-Lepe, J. A. (2015). Wood impregnation of yeast less for winemaking. *Food Chemistry*, *171*, 212-213.

Palomero, F., Morata, A., Benito, S., Calderón, F., Suárez-Lepe, J. A. (2009). New genera of yeasts for over-lees aging of red wine. *Food Chemistry*, *112*, 432-441.

Parker, R. (2012). RNA Degradation in *Saccharomyces cerevisiae*. *Genetics*, *191*, 671-702.

Parr, W. V., Green, J. A., White, K. G., and Sherlock, R. R. (2007). The distinctive flavour of New

Zealand Sauvignon blanc: sensory characterization by wine professionals. *Food Quality and Preference*, *21*, 56-64.

Pati, S., Liberatore, M. T., Lamacchia, C., & La Notte, E. (2010). Influence of ageing on lees on polysaccharide glycosyl-residue composition of Chardonnay wine. *Carbohydrate Polymers*, *80*(2), 332–336.

Pérez-magariño, S., Martínez-Lapuente, L., Bueno-Herrera, M., Ortega-Heras, M., Guadalupe, Z., and Ayestarán, B. (2015). Use of commercial dry yeast products rich in mannoproteins for white and rosé Sparkling wine elaboration. *Journal of Agricultural and Food Chemistry*, *63*, 5670-5681.

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. Food Chemistry, 111(2), 447-456.

Pérez-Serradilla, J. A., & Luque de Castro, M. D. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. Food Chemistry, 124(4), 1652-1659.

Perrot, L., Charpentier, M., Charpentier, C., Feuillat, M., and Chassagne, D. (2002). Yeast adapted to wine: nitrogen compounds released during induced autolysis in a model wine. *Journal of Industrial Microbiology and biotechnology*, *29*, 134-139.

Peynaud, E. (1997). The taste of wine: the art and science of wine appreciation. London: Macdonald and Co Ltd.

Pickering, G. J., and Demiglio, P. (2008). The white wine mouthfeel wheel: a lexicon for describing the oral sensations elicited by white wine. *Journal of Wine Research*, *19*, 51-67.

Pérez-Coello, M. S., and Díaz-Maroto, M. C. (2009). Volatile compounds and wine aging. *Wine Chemistry and Biochemistry*, 295-311. Springer, New York.

Pomeranz, Y., and Meloan, C. E. (3rd Edit) (2000). Food analysis: theory and practice. Aspen Publishers, Gaithersburg, USA.

Pozo-Bayón, M. Á., Andujar-Ortiz, I., Alcaide-Hidalgo, J. M., Martín-Álvarez, P. J., & Moreno-Arribas, M. V. (2009). Characterization of Commercial Inactive Dry Yeast Preparations for Enological Use Based

on Their Ability To Release Soluble Compounds and Their Behavior toward Aroma Compounds in Model Wines. *Journal of Agricultural and Food Chemistry*, *57*(22), 10784–10792.

Prado, J. M., Dalmolin, I., Carareto, N. D. D., Basso, R. C., Meirelles, A. J. A., Vladimir Oliveira, J., Batista, E. A. C., and Meireles, M. A. A. (2012). Supercritical fluid extraction of grape seed: process scale-up, extract chemical composition and economic evaluation. *Journal of Food Engineering*, *109*, 249-257.

Puértolas, E., López, N., Condón, S., Álvarez, I., and Raso, J. (2010). Potential applications of PEF to improve red wine quality. *Trends in Food Science & Technology, 21*, 247–55.

Puértolas, E., López, N., Condón, S., Raso, J., and Álvarez, I. Pulsed electric fields inactivation of wine spoilage yeast and bacteria. (2009). *International Journal of Food Microbiology*, *130*, 49–55.

Puértolas, E., Saldaña, G., Álvarez, I., and Raso, J. (2011) Experimental design approach for the evaluation of anthocyanin content of Rosé wines obtained by pulsed electric fields. Influence of temperature and time of maceration. *Food Chemistry, 126*, 1482–87.

Puértolas, E., Saldaña, G., Condón, S., Álvarez, I., and Raso, J. (2009). A comparison of the effect of macerating enzymes and pulsed electric fields technology on phenolic content and colour of red wine. *Journal of Food Science, 74*, 647–C652.

Quirós, M., Gonzalez, R., and Morales, P. (2012). A simple method for total quantification of mannoprotein content in real wine samples. *Food Chemistry*, *134*, 1205-1210.

Ravishankar, S., Zhang, H., and Kempkes, M. L. (2008). Pulsed electric fields. *Food Science and Technology International, 14*, 429–32.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan application. *Reactive and Functional Polymers*, *46*, 1-27.

Reed, G., & Nagodawithana, T. W. (Eds.). (1991). *Yeast Technology* (Second Edition ed.). New York: Van Nostrand Reinhold.

Ribeiro, T., Fernandes, C., Nunes, F. M., Filipe-Ribeiro, L., and Cosme, F. (2014). Influence of the structural features of commercial mannoproteins in white wine protein stabilization and chemical and sensory properties. *Food Chemistry*, *159*, 47-54.

Rice, A. C. (1976). Solid waste generation and by-product recovery potential from winery residues. *American Journal of Enology and Viticulture, 27*, 21-26.

Risvik, E., McEwan, J. A., and Rodbotten, M. (1997). Evaluation of sensory profiling and projective mapping data. Journal of Food Quality and Preference, 8(1), 63-71.

Roach, P. J., Depaoli-roach, A. A., Hurley, T. D., and Tagliabracci, V. S. (2012). Glycogen and its metabolism: some new developments and old themes. *Biochemical Journal*, *441*, 763-787.

Robinson, J. (1994) (ed). The oxford companion to wine. Oxford University Press: New York, USA.

Rodrígues, A., Richardo-Da-Silva, J. M., Lucas, C., and Laureano, O. (2013). Effect of winery yeast lees on Touriga Nacional red wine colour and tannin evoluation. *American Journal of Viticulture and Enology*, *64*, 98-109.

Rodríguez-Bencomo, J. J., Ortega-Heras, M., Pérez-Magariño, S. (2010). Effect of alternative techniques to aging on lees and use of non-toasted oak chips in alcoholic fermentation on the aromatic composition of red wine. *European Food Research & Technology, 230,* 485 -496.

Rodriguez-Nogales, J. M., Fernández-Fernández, E., & Vila-Crespo, J. (2012). Effect of the addition of β-glucanases and commercial yeast preparations on the chemical and sensorial characteristics of traditional sparkling wine. *European Food Research and Technology*, *235*(4), 729–744.

Roustan, J. L., and Sablayrolles, J. M. (2004). Role of trehalose and glycogen in alcoholic fermentation in winemaking condition. *Journal of Wine Research*, *15*, 189-202.

Runnebaum, R. C., Boulton, R. B., Powell, R. L., and Heymann, H. (2011). Key constituents affecting wine body – an exploratory study. *Journal of Sensory Studies*, *26*, 62-70.

Rowe, J. D., Harbertson, J. F., Osborne, J. P., Freitag, M., Lim, J., and Bakalinsky, A. T. (2010). Systematic identification of yeast proteins extracted into model wine during aging on the yeast lees. *Journal of Agricultural and Food Chemistry*, *58*, 2337-2346.

Salmon, J. M., Vuchot, P., Doco, T., and Moutounet, M. (2003). Maintenance and protection of yeast

morphology by contact with wine polyphenols during simulation of wine aging on lees. *Food Microbiology and Safety*, *68*, 1782-1787.

Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., Herderich, M. J., and Smith, P. A. (2006). Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. *Australian Journal of Grape and Wine Research*, *12*(1), 39-49.

Sartini, E., Arfelli, G., Fabiani, A., and Piva, A. (2007). Influence of chips, lees and microoxygenation during aging on the phenolic composition of a red Sangiovese wine. *Food Chemistry*, *104*, 1599-1604.

Sato, M., Suzuki, Y., Hanamure, K., Katoh, I., Yagi, Y., and Otsuka, K. (1997). Winemaking from Koshu variety by the Sur lie method: behaviour of free amino acids and proteolytic activities in the wine. *American Journal of Enology and Viticulture*, *48*, 433-437.

Sereni, A., Osborne, J., and Tomasino, E. (2016). Exploring retro-nasal aroma's influence on mouthfeel perception of Chardonnay wines. *Beverage*, *2,* 1-11.

Segarra, I., Lao, C., López-Tamames, E., and De La Torre-Boronat, M. C. (1995). Spectrophotometric methods for the analysis of polysaccharide levels in winemaking products. *American Journal of Enology and Viticulture*, *46*, 564-570.

Shynkaryk, M. V., Lebovka, N. I., Lanoisellé, J. L., Nonus, M., Bedel-Clotour, C., Vorobiev, E. (2009). Electrically-assisted extraction of bio-products using high pressure disruption of yeast cells, *Saccharomyces cerevisiae*. *Journal of Food Engineering*, *92*, 189-195.

Smits, G. J., Kapteyn, J. C., Ende, H. V. D., and Klis, F. (1999). Cell wall dynamics in yeast. *Current Opinion in Microbiology*, *2*, 348-352.

Soliva-Fortuny, R., Balasa, A., Knorr, D., and Martín-Belloso, O. (2009). Effects of pulsed electric fields on bioactive compounds in foods: A review. *Trends in Food Science & Technology, 20*, 544–56.

Suárez, B. V., Palacios, N. G., Rodríguez, R. M., and Picinelli, A. L. (2005). Influence of yeast strain and aging time on free amino acid changes in Sparkling ciders. *Journal of Agricultural and Food Chemistry*, *53*, 6408-6413.

Suárez, R., Suárez-Lepe, J. A., Morata, A., and Calderón, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry, 102*, 10–21.

Teillet, E., Schlich, P., Urbano, C., Cordelle, S., and Guichard, E. (2010). Sensory methodologies and the taste of water. *Food Quality and Preference*, *21*, 967-976.

Tewari, G., and Juneja, V. K. (2007) (eds.) *Advances in Thermal and Non-Thermal Food Preservation*. 1st ed. Ames, Iowa: Blackwell Pub.

Tao, Y., Wu, D., Zhang, Q, A., and Sun, D. W. (2014) Ultrasound-assisted extraction of phenolics from wine lees: modelling, optimization and stability of extracts during storage. *Ultrasonics Sonochemistry*, *21*, 706-715.

Todd, B., Fleet, G., and Henschke, P. (2000). Promotion of autolysis through the interaction of killer and sensitive yeasts: potential application in Sparkling wine production. *American Journal of Enology and Viticulture*, *51*, 65-72.

Torresi, S., Frangipane, M. T., Garzillo, A. M. V., Massantini, R., & Contini, M. (2014). Effects of a β-glucanase enzymatic preparation on yeast lysis during aging of traditional sparkling wines. *Food Research International*, *55*, 83–92.

Vasserot, Y., Caillet, S., and Maujean, A. (1997). Study of anthocyanin adsorption by yeast lees. Effect of some physicochemical parameters. *American Journal of Enology & Viticulture*, *48,* 433-437.

Vidal, S., Francis, L., Guyot, S., Marnet, N., Kwiatkowski, M., Gawel, R., Cheynier, V., and Waters, E. J. (2003). The mouthfeel properties of grape and apple proanthocyanidins in a wine-like medium. *Journal of the Science of Food and Agriculture*, *83*, 564-573.

Vidal, S., Courcoux, P., Francis, L., Kwiatkowski, M., Gawel, R., Williams, P., Waters, E., and Cheynier, V. (2004). Use of an experimental design approach for evaluation of key wine components on mouthfeel perception. *Food Quality and Preference*, *15*, 209-217.

Vidal, S., Francis, L., Williams, P., Kwiatkowski, M., Gawel, R., Cheynier, V., and Waters, E. (2004). The mouthfeel properties of polysaccharides and anthocyanin in a wine like medium. *Food Chemistry, 85*, 519-525.

Wang, Y. Y. (2014). Characterization of lees and novel uses for yeast lees to create new wine styles. (Unpublished doctoral dissertation). The University of Adelaide, Australia.

Waterhouse, A. L. (2002). Wine phenolics. *Annals of the New York Academy of Science*, *957*, 21-36.

Werner-Washburne, M., Braun, E., Johnston, G. C., and Singer, R. A. (1993). Stationary phase in the

yeast *Saccharomyces cerevisiae*. *Microbiological Review*, *57*, 383.

Wiemken, A., and Dürr, M. (1974). Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. *Archives of Microbiology*, *101*, 45-57.

Williams, D. L. (1997). Overview of (1→3)-β-D-glucan immunobiology. *Mediators of Inflammation*, *6*, 247-250.

Weijman, A. C. M. and Golubev, W. I. (1987). Carbohydrate patterns and taxonomy of yeasts and yeast-like fungi. *Stud Mycol, 30*, 361-371.

Wu, Y., G. S. Mittal, and M. W. Griffiths. (2005). Effect of pulsed electric field on the inactivation of microorganisms in grape juices with and without antimicrobials. *Biosystems Engineering, 90*, 1–7.

Xiao, L., Zeng, X., Chen, Y., and Yang, H. (2004). Analysis of Infra red on dry red after pulsed electric field treatment. *Food Science*, *1*, 152-155.Yanniotis, S., Kotseridis, G., Orfanidou, A., and Petraki, A. (2007). Effect of ethanol, dry extract and glycerol on the viscosity of wine. *Journal of Food Engineering*, *81*, 399-403.

Yin, Y., Liu, X., Zhu, C., Fan, S., and Luo, G. (2006). Effects of high-voltage pulsed electric field on physical properties of fresh Claret. *Food Science*, *11*, 221-224.

Zeng, X. A., Geng, Y. H., Zhang, B. S., and Li, G, J. (2004). *Chinese Patent No. C12H1/16*. Retrieved from https://patents.google.com/patent/CN1635100A/ru.

Zeng, X. A., Yu, S. J., Zhang, L., and Chen, X. D. (2008). The effects of AC electric field on wine maturation. *Innovative Food Science and Emerging Technologies*, *9*, 463-468.

Zhao, J., and Fleet, G. H. (2003). Degradation of DNA during the autolysis of *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology and Biotechnology, 30,* 175-182.

Zhao, J., and Fleet, G. H. (2005). Degradation of RNA during the autolysis of *Saccharomyces cerevisiae* produces predominantly ribonucleotides. *Journal of Industrial Microbiology and Biotechnology*, *32*, 415-423.

Zhao, D., Zeng, X. A., Sun, D. W., and Liu, D. (2013). Effects of pulsed electric field treatment on (+)-catechin-acetaldhyde condensation. *Innovative Food Science and Emerging Technologies*, *20*, 100-105.

Appendix I. Output of results of statistical analysis of total protein concentration in the experiment described in Chapter 4.

Analysis of variance

Variate: Day_0,Day_8,Day_16,Day_24,Day_32,Day_40,Day_48,Day_56

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.370	0.185	0.08	
Block.Subject stratum					
Lees_material	2	7125.656	3562.828	1554.63	<.001
Enzyme	1	3501.455	3501.455	1527.85	<.001
Stirring_Frequency	2	22.893	11.447	4.99	0.013
Lees_material.Enzyme	2	2266.440	1133.220	494.48	<.001
Lees_material.Stirring_Frequency	4	74.513	18.628	8.13	<.001
Enzyme.Stirring_Frequency	2	59.262	29.631	12.93	<.001
Lees_material.Enzyme.Stirring_Frequency	4	50.320	12.580	5.49	0.002
Residual	34	77.920	2.292	1.32	
Block.Subject.Time stratum					
d.f. correction factor 0.7172					
Time	7	2875.975	410.854	235.98	<.001
Time.Lees_material	14	7329.788	523.556	300.72	<.001
Time.Enzyme	7	623.885	89.126	51.19	<.001
Time.Stirring_Frequency	14	214.865	15.348	8.82	<.001
Time.Lees_material.Enzyme	14	2052.459	146.604	84.21	<.001
Time.Lees_material.Stirring_Frequency	28	162.886	5.817	3.34	<.001
Time.Enzyme.Stirring_Frequency	14	63.236	4.517	2.59	0.006
Time.Lees_material.Enzyme.Stirring_Frequency	28	65.991	2.357	1.35	0.151
Residual	252	438.738	1.741		
Total	431	27006.652			

(d.f. are multiplied by the correction factors before calculating F probabilities)

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

Block 1 Subject 15	1.072	s.e. 0.425
Block 1 Subject 16	0.980	s.e. 0.425
Block 2 Subject 15	-1.119	s.e. 0.425
Block 1 Subject 13 Time Day_40	4.105	s.e. 1.008
Block 1 Subject 16 Time Day_0	4.213	s.e. 1.008
Block 2 Subject 3 Time Day_16	3.089	s.e. 1.008
Block 2 Subject 13 Time Day_40	-3.848	s.e. 1.008
Block 2 Subject 14 Time Day_0	3.341	s.e. 1.008
Block 2 Subject 15 Time Day_0	-3.091	s.e. 1.008
Block 3 Subject 14 Time Day_0	-3.540	s.e. 1.008
Block 3 Subject 15 Time Day_48	-3.425	s.e. 1.008

Tables of means

Variate: Day_0,Day_8,Day_16,Day_24,Day_32,Day_40,Day_48,Day_56

Grand mean 11.007

Time	Day_0	Day_8	Day_16	Day_24	Day_32	Day_40	Day_48
	14.789	15.074	10.920	9.105	9.846	11.771	7.292

Time	Day_56
	9.260

Lees_material	Inactivated yeast	Lees	Rehydrated yeast
	14.139	5.271	13.610

Enzyme	0	1
	13.854	8.160

Stirring_Frequency	2	4	8
	10.685	11.128	11.208

Time	Lees_material	Inactivated yeast	Lees
Day_0		13.075	5.783
Day_8		11.411	5.184
Day_16		16.550	4.694
Day_24		12.282	5.513
Day_32		16.431	2.859
Day_40		17.622	7.272
Day_48		11.413	4.103
Day_56		14.329	6.762

Time	Lees_material	Rehydrated yeast
Day_0		25.509
Day_8		28.627
Day_16		11.515
Day_24		9.519
Day_32		10.247
Day_40		10.417
Day_48		6.359
Day_56		6.687

Time	Enzyme	0	1
Day_0		18.092	11.485
Day_8		20.073	10.075
Day_16		15.023	6.816
Day_24		10.973	7.236
Day_32		13.192	6.499
Day_40		13.411	10.130
Day_48		8.832	5.752
Day_56		11.234	7.285

Lees_material	Enzyme	0	1
Inactivated yeast		19.033	9.246
Lees		4.921	5.622
Rehydrated yeast		17.608	9.612

Time	Stirring_Frequency	2	4	8
Day_0		15.517	14.776	14.074
Day_8		15.195	15.811	14.216
Day_16		9.611	11.388	11.760
Day_24		8.316	9.925	9.073
Day_32		9.454	9.098	10.984
Day_40		10.620	11.497	13.194
Day_48		8.094	6.883	6.898
Day_56		8.668	9.647	9.464

Lees_material	Stirring_Frequency	2	4	8

Inactivated yeast	14.252	14.511	13.655
Lees	5.114	5.164	5.536
Rehydrated yeast	12.688	13.710	14.433

Enzyme	Stirring_Frequency	2	4	8
0		13.724	14.301	13.537
1		7.645	7.956	8.879

Time	Lees_material	Enzyme	0	1
Day_0	Inactivated yeast		14.823	11.326
	Lees		5.840	5.726
	Rehydrated yeast		33.614	17.404
Day_8	Inactivated yeast		14.984	7.839
	Lees		5.048	5.320
	Rehydrated yeast		40.188	17.066
Day_16	Inactivated yeast		23.269	9.830
	Lees		4.700	4.689
	Rehydrated yeast		17.101	5.928
Day_24	Inactivated yeast		15.887	8.677
	Lees		5.296	5.730
	Rehydrated yeast		11.737	7.302
Day_32	Inactivated yeast		23.776	9.085
	Lees		1.633	4.085
	Rehydrated yeast		14.166	6.328
Day_40	Inactivated yeast		22.802	12.443
	Lees		6.938	7.607
	Rehydrated yeast		10.493	10.341
Day_48	Inactivated yeast		16.198	6.629
	Lees		3.687	4.519
	Rehydrated yeast		6.609	6.109
Day_56	Inactivated yeast		20.523	8.135
	Lees		6.222	7.303
	Rehydrated yeast		6.957	6.417

Time	Lees_material	Stirring_Frequency	2	4
Day_0	Inactivated yeast		15.045	13.280
	Lees		5.793	5.851
	Rehydrated yeast		25.713	25.196
Day_8	Inactivated yeast		10.970	13.269
	Lees		5.720	4.661
	Rehydrated yeast		28.896	29.502
Day_16	Inactivated yeast		16.202	16.790
	Lees		3.983	5.362
	Rehydrated yeast		8.648	12.011
Day_24	Inactivated yeast		11.786	14.293
	Lees		4.925	5.581
	Rehydrated yeast		8.237	9.902
Day_32	Inactivated yeast		15.333	15.477
	Lees		3.637	2.204
	Rehydrated yeast		9.393	9.613
Day_40	Inactivated yeast		16.256	17.713
	Lees		6.532	7.191
	Rehydrated yeast		9.072	9.588
Day_48	Inactivated yeast		13.820	10.471
	Lees		4.038	3.946
	Rehydrated yeast		6.426	6.233
Day_56	Inactivated yeast		14.601	14.795
	Lees		6.284	6.514
	Rehydrated yeast		5.118	7.631

Time	Lees_material	Stirring_Frequency	8
Day_0	Inactivated yeast		10.898
	Lees		5.706
	Rehydrated yeast		25.618
Day_8	Inactivated yeast		9.995
	Lees		5.171
	Rehydrated yeast		27.483

Day_16	Inactivated yeast	16.656
	Lees	4.738
	Rehydrated yeast	13.885
Day_24	Inactivated yeast	10.767
	Lees	6.032
	Rehydrated yeast	10.419
Day_32	Inactivated yeast	18.481
	Lees	2.738
	Rehydrated yeast	11.734
Day_40	Inactivated yeast	18.898
	Lees	8.094
	Rehydrated yeast	12.591
Day_48	Inactivated yeast	9.949
	Lees	4.324
	Rehydrated yeast	6.419
Day_56	Inactivated yeast	13.591
	Lees	7.489
	Rehydrated yeast	7.312

Time	Enzyme	Stirring_Frequency	2	4	8
Day_0	0		19.186	18.675	16.416
	1		11.848	10.877	11.732
Day_8	0		20.033	21.857	18.331
	1		10.358	9.765	10.102
Day_16	0		13.906	15.846	15.318
	1		5.317	6.929	8.201
Day_24	0		10.121	11.863	10.935
	1		6.512	7.987	7.211
Day_32	0		12.619	12.516	14.440
	1		6.290	5.680	7.529
Day_40	0		13.078	12.768	14.387
	1		8.162	10.227	12.002
Day_48	0		9.759	8.615	8.121
	1		6.430	5.152	5.674
Day_56	0		11.090	12.266	10.346
	1		6.245	7.027	8.582

Lees_material	Enzyme	Stirring_Frequency	2
Inactivated yeast	0		19.931
	1		8.572
Lees	0		4.519
	1		5.709
Rehydrated yeast	0		16.721
	1		8.654

Lees_material	Enzyme	Stirring_Frequency	4
Inactivated yeast	0		19.682
	1		9.340
Lees	0		5.287
	1		5.040
Rehydrated yeast	0		17.933
	1		9.486

Lees_material	Enzyme	Stirring_Frequency	8
Inactivated yeast	0		17.485
	1		9.824
Lees	0		4.955
	1		6.118
Rehydrated yeast	0		18.170
	1		10.695

Time	Lees_material	EnzymeStirring_Frequency	
			2
Day_0	Inactivated yeas	0	18.153
		1	11.938
	Lees	0	4.780
		1	6.806

	Rehydrated yeast	0	34.626
		1	16.800
Day_8	Inactivated yeas	0	14.991
		1	6.949
	Lees	0	4.942
		1	6.498
	Rehydrated yeast	0	40.164
		1	17.628
Day_16	Inactivated yeas	0	24.136
		1	8.269
	Lees	0	4.256
		1	3.711
	Rehydrated yeast	0	13.326
		1	3.970
Day_24	Inactivated yeas	0	15.822
		1	7.751
	Lees	0	4.191
		1	5.660
	Rehydrated yeast	0	10.349
		1	6.125
Day_32	Inactivated yeas	0	22.926
		1	7.741
	Lees	0	1.693
		1	5.580
	Rehydrated yeast	0	13.239
		1	5.548
Day_40	Inactivated yeas	0	22.475
		1	10.037
	Lees	0	6.352
		1	6.711
	Rehydrated yeast	0	10.407
		1	7.737
Day_48	Inactivated yeas	0	19.489
		1	8.151
	Lees	0	3.597
		1	4.478
	Rehydrated yeast	0	6.190
		1	6.661
Day_56	Inactivated yeas	0	21.458
		1	7.744
	Lees	0	6.343
		1	6.226
	Rehydrated yeast	0	5.470
		1	4.766

Time	Lees_material	EnzymeStirring_Frequency	
			4
Day_0	Inactivated yeas	0	15.665
		1	10.896
	Lees	0	6.868
		1	4.833
	Rehydrated yeast	0	33.491
		1	16.902
Day_8	Inactivated yeas	0	17.427
		1	9.111
	Lees	0	5.767
		1	3.556
	Rehydrated yeast	0	42.377
		1	16.628
Day_16	Inactivated yeas	0	23.473
		1	10.108
	Lees	0	5.374
		1	5.350
	Rehydrated yeast	0	18.693
		1	5.330
Day_24	Inactivated yeas	0	17.713
		1	10.872

	Lees	0	5.536
		1	5.626
	Rehydrated yeast	0	12.342
		1	7.462
Day_32	Inactivated yeas	0	23.132
		1	7.823
	Lees	0	1.768
		1	2.639
	Rehydrated yeast	0	12.649
		1	6.577
Day_40	Inactivated yeas	0	22.623
		1	12.802
	Lees	0	7.028
		1	7.355
	Rehydrated yeast	0	8.652
		1	10.525
Day_48	Inactivated yeas	0	15.801
		1	5.142
	Lees	0	3.434
		1	4.459
	Rehydrated yeast	0	6.609
		1	5.857
Day_56	Inactivated yeas	0	21.623
		1	7.968
	Lees	0	6.525
		1	6.504
	Rehydrated yeast	0	8.651
		1	6.610

Time	Lees_material	EnzymeStirring_Frequency	
			8
Day_0	Inactivated yeas	0	10.651
		1	11.146
	Lees	0	5.873
		1	5.538
	Rehydrated yeast	0	32.724
		1	18.511
Day_8	Inactivated yeas	0	12.532
		1	7.457
	Lees	0	4.436
		1	5.906
	Rehydrated yeast	0	38.024
		1	16.942
Day_16	Inactivated yeas	0	22.199
		1	11.113
	Lees	0	4.470
		1	5.006
	Rehydrated yeast	0	19.285
		1	8.485
Day_24	Inactivated yeas	0	14.125
		1	7.409
	Lees	0	6.161
		1	5.904
	Rehydrated yeast	0	12.519
		1	8.319
Day_32	Inactivated yeas	0	25.272
		1	11.691
	Lees	0	1.439
		1	4.036
	Rehydrated yeast	0	16.609
		1	6.858
Day_40	Inactivated yeas	0	23.307
		1	14.490
	Lees	0	7.434
		1	8.754
	Rehydrated yeast	0	12.420
		1	12.761

Day_48	Inactivated yeas	0	13.305
		1	6.594
	Lees	0	4.030
		1	4.618
	Rehydrated yeast	0	7.029
		1	5.810
Day_56	Inactivated yeas	0	18.488
		1	8.694
	Lees	0	5.799
		1	9.178
	Rehydrated yeast	0	6.750
		1	7.873

Standard errors of means

Table	Time	Lees_material	Enzyme	Stirring_Frequency
rep.	54	144	216	144
d.f.	180.73	34	34	34
e.s.e.	0.1796	0.1262	0.1030	0.1262

Table	Time	Time	Lees_material	Time
	Lees_material	Enzyme	Enzyme	Stirring_Frequency
rep.	18	27	72	18
e.s.e.	0.3171	0.2589	0.1784	0.3171
d.f.	214.73	214.73	34	214.73
Except when comparing means with the same level(s) of				
Lees_material	0.3110			
d.f.	180.73			
Enzyme		0.2539		
d.f.		180.73		
Stirring_Frequency				0.3110
d.f.				180.73

Table	Lees_material	Enzyme	Time	Time
	Stirring_Frequency	Stirring_Frequency	Lees_material	Lees_material
			Enzyme	Stirring_Frequency
rep.	48	72	9	6
e.s.e.	0.2185	0.1784	0.4484	0.5492
d.f.	34	34	214.73	214.73
Except when comparing means with the same level(s) of				
Lees_material.Enzyme			0.4398	
d.f.			180.73	
Lees_material.Stirring_Frequency				0.5387
d.f.				180.73

Table	Time	Lees_material	Time	
	Enzyme	Enzyme	Lees_material	
	Stirring_Frequency	Stirring_Frequency	Enzyme	
			Stirring_Frequency	
rep.	9	24	3	
e.s.e.	0.4484	0.3090	0.7767	
d.f.	214.73	34	214.73	
Except when comparing means with the same level(s) of				
Enzyme.Stirring_Frequency	0.4398			
d.f.	180.73			
Lees_material.Enzyme.Stirring_Frequency			0.7618	
d.f.			180.73	

Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)

Standard errors of differences of means

Table	Time	Lees_material	Enzyme	Stirring_Frequency
rep.	54	144	216	144
d.f.	180.73	34	34	34
s.e.d.	0.2539	0.1784	0.1457	0.1784

Table	Time	Time	Lees_material	Time
	Lees_material	Enzyme	Enzyme	Stirring_Frequency
rep.	18	27	72	18
s.e.d.	0.4484	0.3661	0.2523	0.4484
d.f.	214.73	214.73	34	214.73
Except when comparing means with the same level(s) of				
Lees_material	0.4398			
d.f.	180.73			
Enzyme		0.3591		
d.f.		180.73		
Stirring_Frequency				0.4398
d.f.				180.73

Table	Lees_material	Enzyme	Time	Time
	Stirring_Frequency	Stirring_Frequency	Lees_material	Lees_material
			Enzyme	Stirring_Frequency
rep.	48	72	9	6
s.e.d.	0.3090	0.2523	0.6342	0.7767
d.f.	34	34	214.73	214.73
Except when comparing means with the same level(s) of Lees_material.Enzyme				
			0.6220	
d.f.			180.73	
Lees_material.Stirring_Frequency				0.7618
d.f.				180.73

Table	Time	Lees_material	Time	
	Enzyme	Enzyme	Lees_material	
	Stirring_Frequency	Stirring_Frequency	Enzyme	
			Stirring_Frequency	
rep.	9	24	3	
s.e.d.	0.6342	0.4370	1.0984	
d.f.	214.73	34	214.73	
Except when comparing means with the same level(s) of Enzyme.Stirring_Frequency				
	0.6220			
d.f.	180.73			
Lees_material.Enzyme.Stirring_Frequency				
			1.0773	
d.f.			180.73	

Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)

Least significant differences of means (5% level)

Table	Time	Lees_material		
			Enzyme	Stirring_Frequency
rep.	54	144	216	144
d.f.	180.73	34	34	34
l.s.d.	0.5306	0.3626	0.2960	0.3626

Table	Time	Time	Lees_material	
	Lees_material			Time
		Enzyme	Enzyme	Stirring_Frequency
rep.	18	27	72	18
l.s.d.	0.9361	0.7643	0.5128	0.9361
d.f.	214.73	214.73	34	214.73
Except when comparing means with the same level(s) of				
Lees_material	0.9190			
d.f.	180.73			
Enzyme		0.7504		
d.f.		180.73		
Stirring_Frequency				
				0.9190
d.f.				180.73

Table	Lees_material		Time	Time
		Enzyme		
	Stirring_Frequency			
		Stirring_Frequency		
			Lees_material	
				Lees_material
			Enzyme	Stirring_Frequency
rep.	48	72	9	6
l.s.d.	0.6280	0.5128	1.3238	1.6213
d.f.	34	34	214.73	214.73
Except when comparing means with the same level(s) of				
Lees_material.Enzyme				
			1.2996	
d.f.			180.73	
Lees_material.Stirring_Frequency				
				1.5917
d.f.				180.73

Table	Time	Lees_material		
			Time	
	Enzyme	Enzyme	Lees_material	
	Stirring_Frequency			
		Stirring_Frequency		
			Enzyme	
			Stirring_Frequency	
rep.	9	24	3	
l.s.d.	1.3238	0.8881	2.2929	
d.f.	214.73	34	214.73	
Except when comparing means with the same level(s) of				
Enzyme.Stirring_Frequency				
	1.2996			
d.f.	180.73			
Lees_material.Enzyme.Stirring_Frequency				
			2.2511	
d.f.			180.73	

Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)

Appendix II. Output of results of statistical analysis of total protein concentration in the experiment described in Chapter 5.

Analysis of variance

Variate: Day_0,Day_2,Day_4,Day_6,Day_8,Day_12,Day_16,Day_20,Day_24,Day_32,Day_40,Day_48,Day_56

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replicate stratum	2	5.135	2.567	1.06	
Replicate.Subject stratum					
Lees_material	3	4480.357	1493.452	617.09	<.001
Enzyme	2	2829.052	1414.526	584.48	<.001
Lees_material.Enzyme	6	263.504	43.917	18.15	<.001
Residual	22	53.243	2.420	0.97	
Replicate.Subject.Time stratum					
d.f. correction factor 0.4525					
Time	12	54695.184	4557.932	1825.04	<.001
Time.Lees_material	36	22952.972	637.583	255.29	<.001
Time.Enzyme	24	3937.149	164.048	65.69	<.001
Time.Lees_material.Enzyme	72	779.573	10.827	4.34	<.001
Residual	288	719.263	2.497		
Total	467	90715.433			

(d.f. are multiplied by the correction factors before calculating F probabilities)

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

Replicate 1 Subject 7 Time Day_2	3.631	s.e. 1.240
Replicate 1 Subject 7 Time Day_20	-3.901	s.e. 1.240
Replicate 1 Subject 10 Time Day_2	3.743	s.e. 1.240
Replicate 1 Subject 12 Time Day_2	-5.728	s.e. 1.240
Replicate 2 Subject 2 Time Day_16	4.125	s.e. 1.240
Replicate 3 Subject 3 Time Day_0	3.958	s.e. 1.240
Replicate 3 Subject 7 Time Day_20	3.720	s.e. 1.240
Replicate 3 Subject 10 Time Day_2	-5.409	s.e. 1.240
Replicate 3 Subject 12 Time Day_2	6.192	s.e. 1.240

Tables of means

Variate: Day_0,Day_2,Day_4,Day_6,Day_8,Day_12,Day_16,Day_20,Day_24,Day_32,Day_40,Day_48,Day_56

Grand mean 14.439

Time	Day_0	Day_2	Day_4	Day_6	Day_8	Day_12	Day_16
	28.695	38.640	25.909	24.563	12.333	13.752	9.992

Time	Day_20	Day_24	Day_32	Day_40	Day_48	Day_56
	5.492	6.704	5.877	7.123	4.357	4.277

Lees_material	NI	NR	PH	PL
	10.867	11.933	18.101	16.857

Enzyme	0	1	7
	17.375	14.585	11.358

Time	Lees_material	NI	NR	PH	PL
Day_0		10.622	14.365	46.488	43.305
Day_2		12.604	26.772	60.247	54.937
Day_4		13.778	25.444	33.702	30.710
Day_6		17.726	20.899	30.961	28.667
Day_8		12.404	12.648	13.408	10.870
Day_12		15.241	12.042	14.009	13.717
Day_16		14.333	9.829	8.127	7.680
Day_20		8.500	4.363	4.920	4.184
Day_24		8.891	4.754	6.891	6.278
Day_32		5.447	6.437	6.290	5.332
Day_40		8.643	8.429	4.696	6.723
Day_48		6.755	4.061	3.007	3.605
Day_56		6.330	5.083	2.560	3.136

Time	Enzyme	0	1	7
Day_0		30.451	29.214	26.420
Day_2		46.706	40.414	28.800
Day_4		33.522	27.720	16.485
Day_6		32.381	25.773	15.535
Day_8		15.996	11.418	9.584
Day_12		19.989	12.429	8.839
Day_16		11.534	9.815	8.628
Day_20		5.967	5.349	5.158
Day_24		6.973	6.595	6.544
Day_32		6.015	6.034	5.580
Day_40		8.459	6.336	6.574
Day_48		3.383	4.822	4.867
Day_56		4.505	3.683	4.644

Lees_material	Enzyme	0	1	7
NI		13.475	9.941	9.185
NR		14.718	11.772	9.309
PH		21.356	19.216	13.730
PL		19.952	17.410	13.209

Time	Lees_material	Enzyme	0	1	7
Day_0	NI		12.282	9.628	9.955
	NR		16.034	13.688	13.374
	PH		47.815	48.626	43.025
	PL		45.675	44.916	39.325
Day_2	NI		17.611	11.453	8.748
	NR		36.761	26.124	17.432
	PH		70.353	64.056	46.333
	PL		62.099	60.024	42.687
Day_4	NI		17.551	13.658	10.126
	NR		33.365	27.635	15.333
	PH		42.215	36.981	21.912
	PL		40.958	32.605	18.567

Day_6	NI	26.473	15.609	11.095
	NR	27.142	22.617	12.938
	PH	40.143	34.607	18.132
	PL	35.765	30.261	19.974
Day_8	NI	16.458	10.291	10.464
	NR	13.827	10.916	13.202
	PH	18.549	13.748	7.927
	PL	15.150	10.716	6.744
Day_12	NI	24.316	11.645	9.761
	NR	16.434	10.737	8.955
	PH	20.400	13.602	8.024
	PL	18.804	13.731	8.616
Day_16	NI	16.019	13.808	13.171
	NR	11.985	9.006	8.496
	PH	8.937	9.184	6.261
	PL	9.193	7.261	6.585
Day_20	NI	10.002	8.979	6.519
	NR	4.487	4.222	4.378
	PH	4.251	5.792	4.716
	PL	5.130	2.403	5.019
Day_24	NI	9.851	8.423	8.398
	NR	4.556	4.733	4.973
	PH	7.589	6.737	6.348
	PL	5.894	6.485	6.456
Day_32	NI	5.366	5.464	5.511
	NR	6.801	6.741	5.770
	PH	6.641	6.583	5.644
	PL	5.254	5.348	5.394
Day_40	NI	9.112	6.896	9.920
	NR	9.483	8.210	7.594
	PH	5.940	3.214	4.935
	PL	9.299	7.022	3.848
Day_48	NI	4.522	7.967	7.777
	NR	4.074	4.683	3.425
	PH	2.128	3.759	3.135
	PL	2.807	2.878	5.130
Day_56	NI	5.616	5.417	7.956
	NR	6.381	3.720	5.148
	PH	2.671	2.919	2.090
	PL	3.351	2.676	3.380

Standard errors of differences of means

Table	Time	Lees_material	Enzyme	Time Lees_material
rep.	36	117	156	9
s.e.d.	0.3725	0.2034	0.1761	0.7441
d.f.	130.32	22	22	146.55
Except when comparing means with the same level(s) of				
Lees_material				0.7450
d.f.				130.32

Table	Time Enzyme	Lees_material Enzyme	Time Lees_material Enzyme
rep.	12	39	3
s.e.d.	0.6444	0.3523	1.2888
d.f.	146.55	22	146.55
Except when comparing means with the same level(s) of			
Enzyme	0.6452		
d.f.	130.32		
Lees_material.Enzyme			
			1.2903
d.f.			130.32

Correction factors have been applied to residual d.f.(see analysis-of-variance table for details)